THE EFFECT OF GENTIAN VIOLET ON PROTOZOA AND ON TISSUES GROWING IN VITRO, WITH ESPECIAL REFERENCE TO THE NUCLEUS.*

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PLATE 26.

In the original communication of Churchman[1] on the selective bactericidal action of gentian violet, published two years ago, the suggestion was made that similar studies should be carried out on the effect of the dye on growing tissue. This communication contains the report[2] of work undertaken in that direction. The work was primarily undertaken to settle two questions raised by the observations on the effect of gentian violet on bacteria. These two questions were (a) whether the nucleus of a single-celled organism could be stained during life, and (b) whether such a stained organism was capable of further reproduction.

It was noticed early in the gentian violet studies that motile organisms not killed by the stain (violet-negative organisms) retained their motility even though deeply stained; and that these stained violet-negative organisms when transplanted to agar slants grew equally well with the control smears of the unstained bacteria. The retention of motility by the stained organisms might be explained as a survival phenomenon; and the growth of the transplants made from the stained specimens might be regarded as arising, not from the organisms in the smear which had taken the stain but from the few in the smear which had escaped it. It

* The experimental work on which this communication is based was presented by the author in a graduation thesis required for the degree of Doctor of Medicine in Yale University. Received for publication, July 28, 1914.

[1] Churchman, J. W., *Jour. Exper. Med.*, 1912, xvi, 221.

[2] Churchman, J. W., and Russell, D. G., *Proc. Soc. Exper. Biol. and Med.*, 1913–14, xi, 120. This was the preliminary report of this work.

seemed altogether likely, from other observations, that this explanation was not the correct one, and that the violet-negative organisms actually took the stain during life. Still, definite proof was lacking that gentian violet in these experiments was acting as a true vital stain. To furnish this proof and to investigate the further problem (raised but not solved by the experiments with bacteria) as to whether the violet dye stained the nucleus of the protoplasm, experiments have been done with protozoa, adult living tissue, and embryonic living tissue.

THE EFFECT OF GENTIAN VIOLET ON PROTOZOA.

The organisms first used for this purpose were paramecia from a pedigreed race furnished by Professor Woodruff. The effect of the dye was investigated in two ways. In the first experiments the organism was stained and then transplanted. In the second series the organism was grown in media containing the dye.

TECHNIQUE.

In the first series of experiments the following technique was used: A single paramecium was placed in a watch-glass containing stain of varying dilutions (1 to 200, 1 to 10,000, and 1 to 100,000) and stained for half a minute, one minute, and five minutes, respectively. The organism was then recovered with a fine pipette, washed thoroughly in two or three washings of distilled sterile water, transplanted to a medium of 0.025 per cent. beef broth (Woodruff and Baitsell,[3] 1911) which had been inoculated with *Bacillus prodigiosus*. The paramecia were studied in drops of this medium on depression slides, which were kept in large covered glass dishes with water in the bottom to prevent evaporation.

In these experiments the organism was found to stain deeply in all the gentian violet dilutions used. The rapidity with which the organisms took up the stain and lost their motility was in direct ratio to the concentration of the dye. In the strongest solution the organism became promptly stained and motility was rapidly lost. In the weaker solutions the paramecia swam about actively

[3] Woodruff, L. L., and Baitsell, G. A., *Jour. Exper. Zoöl.*, 1911, xi, 135.

and normally and it was quite easy to watch the absorption of the dye by both cytoplasm and nucleus. This staining of cytoplasm and nucleus took place while motility was still retained and while the cilia were still whipping. The dye, however, soon caused a retardation of motility and later a cessation; and then the organisms became so deeply stained that no structure could be made out. Such an organism when transplanted did not resume its activity and in a short time broke up into unrecognizable debris.

In the second series of experiments the effect of the dye was studied by transplanting the paramecia into media containing gentian violet.

The technique was as follows: A single paramecium was planted on a depression slide in a drop of 0.025 per cent. beef broth, to which gentian violet in varying strengths (1 to 400 up to 1 to 10,000,000) was added. These drops had been previously inoculated with *Bacillus prodigiosus*. Evaporation was prevented as before.

The effect of the dye in these experiments was sharp and constant. In dilutions stronger than 1 to 500,000 the nucleus as well as the cytoplasm soon became distinctly stained and the cell outline sharply defined. This took place while active motility, both rotatory and progressive, was still retained, and while the cilia were still whipping violently. In a short time, however, the motility diminished and the organism finally came to a standstill. The cilia continued for some time to wave but later these also stopped. The organism then gradually swelled until the cell membrane ruptured, allowing the protoplasm to escape; and the organism either appeared as a deeply stained motionless and structureless mass, or else persisted only as unrecognizable debris. In strengths of 1 to 100,000 cessation of motility occurred in a few minutes. In 1 to 500,000 dilutions some of the organisms were still motile forty-eight hours after immersion but none of them survived after three days and none divided. In 1 to 1,000,000 dilutions fission was always delayed and in a number of cases prevented, though some organisms reproduced slowly and a few were apparently faintly stained in the dividing stage. In strengths weaker than 1 to 1,000,000 there were suggestions of staining in a few instances, but no apparent inhibition of motility or reproduction.

A number of similar observations were made on oxytricha. The technique was similar to that used with paramecia. The results with this organism, though in general the same as those just recorded for paramecia, were not quite so well defined and constant, owing to the fact that this organism is more difficult to handle without injury.

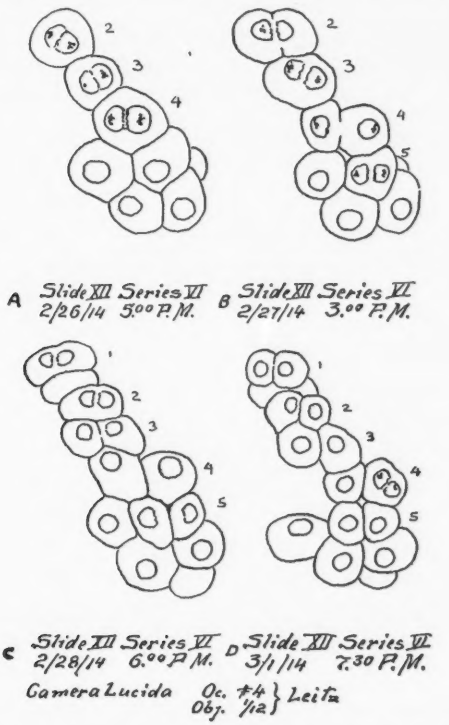
There was no question that the nucleus in these experiments with paramecium and oxytricha was deeply stained, while both organism and cilia were actively motile; and if motility be regarded as a certain indication of life the observations warrant the conclusion that gentian violet is a true vital nuclear stain. We were unable, however, to observe cell division in a definitely stained organism; and the motility in these experiments might well be interpreted as a survival phenomenon in dead or dying organisms. The nature of the experiments was therefore changed and the effect of the dye on growing animal tissue was investigated.

THE EFFECT OF GENTIAN VIOLET ON ADULT FROG TISSUE GROWING IN VITRO.

In the first series of experiments connective tissue from the adult frog was grown in frog plasma, according to the technique described by Harrison.[4] The effect of the gentian violet was studied by adding it to the plasma into which the tissue plants were made. The dye had no effect on the clotting of the plasma, as was to be expected from the observations of Churchman on the effects of gentian violet on enzymes.[5] The connective tissue was found to grow rather poorly, particularly in the early experiments, in which very strong dilutions of stain were used. In preparing a series of connective tissue plants from the fascia of the upper abdominal muscles, pericardium was accidentally included; and the endothelial cells in these transplants showed definite growth, while the connective tissue cells were apparently inhibited by the dye. In the first experiments the plasma contained gentian violet in a dilution of 1 to 2,000. Definite endothelial growth occurred even in this strength but in all subsequent experi-

[4] Harrison, R. G., *Anat. Rec.*, 1906–08, i, 209; *Jour. Exper. Zoöl.*, 1910, ix, 787.
[5] Churchman, *Proc. Soc. Exper. Biol. and Med.*, 1913–14, xi, 54.

ments a 1 to 20,000 dilution was used. Here the growth of the endothelium was active, keeping pace with the controls. In some cases, indeed, the growth in the stained media seemed to outstrip that in the controls. This growth occurred in spite of the fact that the tissue plant, soon after being placed in the dye-containing plasma, became deeply stained. That this tissue plant was stained while alive and growing was demonstrated by transplanting a piece of the tissue which had grown in the dye-containing plasma to the unstained plasma, where the growth of the deeply stained ex-plant continued. These observations made it clear that the cell nucleus


A Slide XII Series VI 2/26/14 5.00 P.M.
B Slide XII Series VI 2/27/14 3.00 P.M.
C Slide XII Series VI 2/28/14 6.00 P.M.
D Slide XII Series VI 3/1/14 7.30 P.M.
Camera Lucida Oc. #4 Obj. 1/12 Leitz

TEXT-FIG. 1. Diagrams made on four succeeding days of endothelial cells of the pericardium of an adult frog, grown in plasma containing gentian violet 1 : 20,000, showing that cell division was observed from beginning to completion. Figures (1, 2, 3, 4, and 5) have been placed so that it is possible to follow the individual cells in the four diagrams.

itself was stained and that this staining was vital and did not interfere with growth. The staining of the plasma and nucleus is well shown in figure 1.

In a number of specimens it was possible to follow the division of these stained nuclei and to observe the whole process with the oil immersion lens (text-figure 1). In these early experiments with adult tissue, however, clear karyokinetic figures were not seen. In one specimen a piece of peritoneum from the female frog was planted in stained plasma. The cilia were observed to remain actively motile for a week, and active growth of the transplant produced new ciliated endothelium. In another experiment the tissue was kept alive and growing for over two weeks in the stained plasma by transplanting the tissue every three or four days into freshly stained plasma.

In no experiment on the growth of living tissue in stained plasma did an infection ever occur, while in the controls infection with bacteria was not infrequent. We were also struck by the fact that endothelial cells grew much more readily in the presence of the dye than connective tissue cells, and we are inclined to the view that we are dealing here with a selective action not unlike that exercised by this dye on bacteria.

Another fact brought out by these experiments was the power of the growing animal cells to change the dye so that its color disappears. That a similar change takes place in the animal body was pointed out by Churchman and Herz in a previous communication.[6] It was there shown that large amounts of gentian violet injected into the ear vein of the rabbit soon disappeared from the blood; and that the mucosa of the tongue and lips, though at first deeply stained, in a short time (about forty-eight hours) lost their violet color. Animals killed a few days after the injection showed no trace of the dye, nor did it appear as such in the urine.

THE EFFECT OF GENTIAN VIOLET ON EMBRYONIC FROG TISSUE GROWING IN VITRO.

In this series of experiments, tadpoles of *Rana silvatica* and *Rana pipiens* in the pre-gill and gill stages were used. The effect of

[6] Churchman, J. W., and Herz, L. F., *Jour. Exper. Med.*, 1913, xviii, 579.

gentian violet was studied in preparations of the tissues planted in stained plasma prepared according to the following technique.

A tadpole was washed thoroughly in sterile distilled water and then placed in sterile 0.3 per cent. saline solution. Under a Zeiss binocular a small incision was made in the skin and the flap turned back. In *Rana silvatica* the skin could not be separated from the underlying connective tissue there, so that cultures made from pieces of the skin of the flap contained both epithelium and mesenchyme. In *Rana pipiens* the skin could be easily separated from the connective tissue and so pure cultures of epithelium and mesenchyme were made. In tadpoles in the gill stage the tissue cultures were made from pieces of the gills (which could easily be cut off), from the tail (where the pigment is practically absent), and from the heart. The tissue plants were all made into plasma containing stain of varying dilutions (1 to 4,000 up to 1 to 20,000); and controls were simultaneously made in unstained plasma.

The embryonic tissues remained alive and grew in the presence of the stain. In the mixed cultures of epithelium and mesenchyme the epithelial cells grew rapidly, while the mesenchyme was apparently retarded by the action of the dye. The same selective action of the stain was also brought out in the pure cultures of epithelium and mesenchyme. In the former, growth was active and rapid, while in the latter growth was slight and in some cases entirely prevented. In the preparations made from the gills, both the epithelial and endothelial cells grew well in the stained plasma; while in the preparations from the heart pure cultures of endothelial cells were obtained.

In one series of epithelial tissue cultures, ciliated epithelium was present and the cilia remained actively motile for over two weeks in the presence of gentian violet in the dilution of 1 to 20,000. The growth of epithelium in stained plasma (dilution of 1 to 4,000) was not as active and rapid as in the weaker dilutions used. In the weaker dilutions growth of epithelium and endothelium was active and the nuclei were slightly stained. These stained nuclei did not stand out as clearly and distinctly as those in the more slowly growing adult endothelium but were definitely violet in color. In a small series of experiments, made very carefully

with this point in view, we have observed the division of the definitely stained nuclei and the presence of karyokinetic figures in these dividing stained nuclei (figure 1).

In the experiments with embryonic tissue the effect of the growing cells on the stain was much more marked than in the case of growing adult tissue. In twenty-four hours the color of the stain was decreased very markedly and in dilutions of 1 to 20,000 the violet color was almost entirely removed by the end of four days.

These experiments prove that gentian violet penetrates by diffusion the living cell and its living nucleus; for the staining of animal cells which subsequently grow in a normal manner cannot be interpreted as a survival phenomenon, and the observation of the staining of the karyokinetic figures in these growing cells demonstrates that the dye penetrates into the nucleus itself.

CONCLUSIONS.

1. Gentian violet may be regarded as a true vital nuclear stain.

2. Embryonic and adult tissue of the frog will grow *in vitro* in the presence of gentian violet of a far stronger dilution than that necessary to kill many bacteria. In these experiments, for example, successful tissue growths were obtained when gentian violet 1 to 20,000 was used, yet *Bacillus subtilis* will not grow in 1 to 100,000 dilution and grows very badly in 1 to 1,000,000 dilution. This fact may simplify the technique of the growth of certain tissues by eliminating the risk of bacterial contamination.

3. The use of stains in the plasma in which tissue is grown will probably facilitate the study of nuclear growth.

4. Gentian violet appears to have a certain selective action on tissue similar to that exercised by the dye on bacteria. Certain observations made last year in this laboratory (too few to serve as more than a suggestion) seem to indicate that another dye (methylene blue) acted as a stimulant to the growth of connective tissue. These leads should be followed out and the effect of various stains studied in the hope of discovering dyes which will exercise a sharp selective action on growing tissue.

5. The growth of animal cells in a strength of dye much more than sufficient to kill many pathogenic organisms lends encourage-

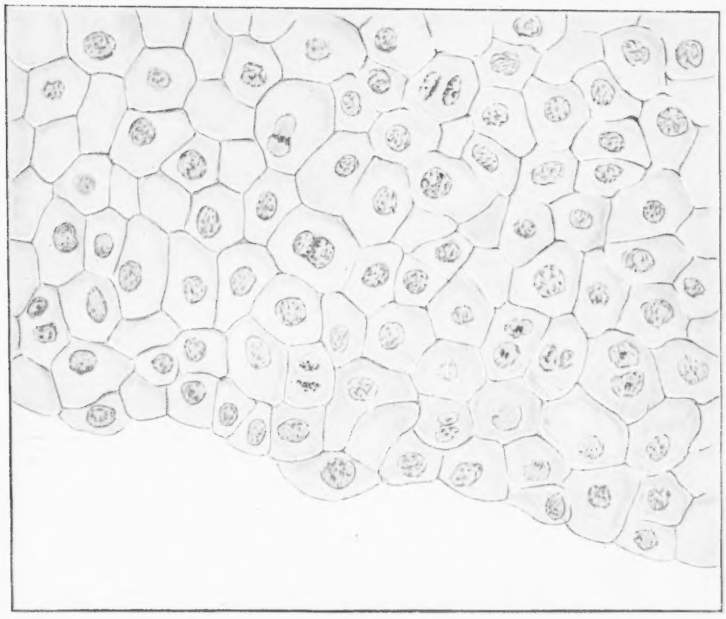


FIG. 1.

(Russell: Effect of Gentian Violet on Protozoa.)

ment to efforts now being made in this laboratory to apply the observations on the bactericidal effect of gentian violet and allied stains to the treatment of disease. Moreover, the ability of growing cells to rid themselves of this dye would indicate that it may be possible to apply the dye to infected tissue and count on the cells to eliminate the material after its work had been done.

EXPLANATION OF PLATE 26.

FIG. 1. New growth of epithelial cells, from the skin of the tail of *Rana pipiens* in the gill stage, growing in stain-containing plasma (gentian violet 1:4,000). The nuclei are distinctly stained, showing dividing forms with suggestion of staining of the chromosomes. The cell outlines are very distinct and the cytoplasm is stained. Drawn in natural colors with the camera lucida, ocular No. 4, objective ⅛, Leitz.

THE EFFECT OF VARIOUS TISSUE EXTRACTS UPON THE GROWTH OF ADULT MAMMALIAN CELLS IN VITRO.*

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PLATES 27 TO 31.

It was shown in a previous communication[1] that the extent of growth of adult mammalian tissue is to a large extent dependent upon the nature of the plasmatic medium used. It then became necessary to investigate the effects of various tissue extracts upon the growth, for it is possible that the variations in the plasmata of different animals might in part be dependent upon the secretions of various glands. There is already considerable clinical evidence that glandular secretions affect the growth of various tissues *in vivo*. Carrel has also shown[2] that the growth of tissue *in vitro* is stimulated to a marked degree by certain tissue extracts, especially, in the case of the chick, by the extract of chick embryo, but the presence or absence of any specific action of the extracts on different tissues has not been investigated. This communication will deal with the effects of various extracts of adult tissue upon the growth of cells obtained from adult mammals.

TECHNIQUE.

In all cases the plasma and tissues of adult rabbits were used both for cultural purposes and for preparing the extracts. The cultures were prepared by Carrel's technique in the manner I have previously described,[3] but after the piece of tissue had been placed in the

* The expenses connected with this work were defrayed by a grant from the London Hospital Research Fund. Received for publication, August 17, 1914.

[1] Walton, A. J., *Proc. Roy. Soc. London*, 1914, series B, lxxxvii, 452.

[2] Carrel, A., and Burrows, M. T., *Jour. Exper. Med.*, 1911, xiv, 244. Carrel, A., *idem*, 1912, xv, 516; 1913, xvii, 14; 1913, xviii, 287.

[3] Walton, A. J., *Jour. Path. and Bacteriol.*, 1914, xviii, 319.

plasma on the sterile cover-slip, a little of the fluid extract, equal in quantity to about one half the amount of plasma, was added with a sterile pipette and the fluids were mixed with a cataract knife. Almost immediately after the addition of the tissue extract the plasma coagulated. The preparation was then inverted over the cell slide and sealed in the usual way. In all cases, both in the primary and in the subcultures, an equal number of controls were made, the tissue in this case being grown in simple plasma. This was essential in every case for, as I have previously shown, the amount of growth varies considerably with the use of different specimens of plasma. It was also necessary to have controls in which the plasma was mixed with some inert fluid so that the changes in growth brought about by dilution should not be confounded with those caused by the addition of the extract. For this purpose a certain number of preparations were made in which the plasma was mixed with about one half of its volume of Ringer's fluid. Under these conditions it was found, as Carrel has previously shown,[4] that growth was somewhat accelerated. Allowance was made for this acceleration in deducing the results of the experiments.

Preparation of the Extracts.—The extracts were prepared by cutting or grinding up the given tissue in sterile Ringer's fluid, leaving the mixture to stand for a short while, and then centrifugalizing it. The supernatant fluid was then pipetted off and used as the extract. The fine division of the tissue in a sterile condition presented some difficulties. In the case of soft tissues such as the testicle, liver, spleen, and kidneys these difficulties were overcome by the use of the following instrument.[5] A brass syringe of a capacity of about seven cubic centimeters was made, the piston of which had two cross bars projecting from its face. These cross bars were made to sink in flush with the rest of the face of the piston, but were kept projecting by means of springs. When so projecting they acted as cutters, but when the piston was pressed down they could sink into it and the face of the piston was then smooth. The nozzle of the syringe was made to unscrew and between it and the barrel was inserted a perforated steel plate. The perforations were ta-

[4] Carrel, A., and Burrows, M. T., *Jour. Exper. Med.*, 1911, xiii, 562.

[5] I am indebted to Mr. H. S. Souttar for assistance in devising this instrument.

pered so that the narrow openings were nearest the piston and the edges on this surface were therefore sharp. The syringe was sterilized and a piece of the required tissue inserted into it. On rotating the piston the cross bars forced the tissue round so that it was cut on the perforations in the steel plate. The piston being at the same time slowly pushed home, the cut fragments of the tissue were forced through the plate. The cross bars sinking in as the piston was forced home, no dead space was left and all the tissue was thus used up. A small amount of Ringer's fluid was now sucked up into the syringe and formed an emulsion with the cut tissue. The mixture was then ejected into a sterile glass tube, corked, and, after standing for a short while, centrifugalized. For the larger pieces of tissue the small mincing machine devised by Haaland[6] would be equally efficacious.

Certain tissues, such as muscle, were too tough to be cut up by this instrument; they were therefore minced as small as possible with sterile scissors in Ringer's fluid and then centrifugalized.

The experiments were carried out in two groups. In the first group the animal was anesthetized, blood removed from the carotid artery, and the tissue required for making the extract then cut out. Small pieces of the viscera which were to be cultivated were then removed and placed in a sterile bowl of Ringer's fluid. By this means cultures were made of several tissues in the same extract which was autogenous to the tissues. 805 cultures were made in this group.

In the second group an animal was killed on a certain day, several of its organs were removed, and extracts made from them. The extracts were kept on ice for twenty-four hours and then centrifugalized. Subsequently one viscus was removed from another animal and cultured with all the extracts. By this means cultures were made of one tissue in several extracts which were homogenous to the tissue. Two hundred and sixteen cultures were made in this group.

This second group served not only as a control to the first group and thereby decreased the possibility of experimental error, but also served to show whether there was any alteration when homogenous instead of autogenous extracts were used.

[6] Haaland, M., *Berl. klin. Wchnschr.*, 1907, xliv, 713.

The results obtained by the former method will be first considered.

SPLEEN EXTRACT.

In this investigation 238 cultures were made, the effect of the extract being tried on the growth of spleen, thyroid, testicle, liver, and kidney. It was found that the tissues reacted somewhat differently, so that the effects on each tissue must be described.

Spleen.—Of this tissue fifty-eight cultures were made and the results were found to be constant. Even in the early stages the emigration of cells was somewhat more marked in the case of the cultures with the extract than in the controls. At the end of twenty-four hours the number of round cells in the plasma was distinctly greater and they had passed further into the medium. It is doubtful, however, if these cells should be described as parenchymatous cells. They appear to be polynuclear and mononuclear blood cells, and never in any of the preparations have mitotic figures been demonstrable in them, either in the controls or in the preparations with the extracts. The presence of these cells in large numbers to a certain extent obscures the changes of true growth and gives rise to some difficulty in the comparison of any two specimens. They must not be confounded with other cells which appear after two or three days. These are much larger, cuboidal in shape, and grow in solid masses from the edge of the tissues. They have a well defined nucleus with a wide zone of protoplasm. They are probably parenchymatous in nature and arise from the spleen pulp. They are quite distinct from the radiating, branching, and more deeply staining connective cells which on the third or fourth day begin to grow into them from the edge of the tissue. In the preparations to which spleen extract had been added the growth of these cells was always more marked than in the controls, so that whereas on the fourth day specimens grown in simple plasma would often show only irregular patches of these cells the corresponding specimens with the extract would not infrequently show a wide mosaic-like sheet as wide as, or wider than, the original piece of tissue. After four or five days the growth of cells of the connective tissue type was also more advanced in the stimulated specimens. The increase was not very marked, but was constant in all cases. Not only was the

growth of this type of cell more extensive, but it commenced at an earlier date so that they were seen on the second or third day, whilst in the controls they were not definite until the third or even the fourth day. In the subcultures both the parenchymatous and connective cells were more marked in the specimens with the extract for the first subculture, but in the later subcultures the parenchymatous cells were overgrown by the connective tissue type, the growth of which, however, was always more extensive in the specimens containing the extract.

Thyroid.—Fifty-four cultures were made of this tissue. In some of the earlier experiments no growth occurred with the extract, but in all these there was very slight growth in the controls. In the later experiments the growth was much more marked in the preparations with the extract than in the controls. The cells grew rapidly from the edge of the tissue and were mainly of the parenchymatous type, so that after five or six days large solid masses of cuboidal cells were seen projecting into the surrounding medium. Although the greater number of the cells were of the parenchymatous type there was also an increase of the connective tissue cells, these in all cases being more abundant than in the controls. In the case of the controls the majority of the cells were of the connective tissue type, there being very few parenchymatous cells present.

When subcultures were made the cells even of the stimulated specimens became more and more of the connective tissue type. That is to say, when life is prolonged the connective tissue cells, both in stimulated and unstimulated specimens, tend to overgrow the parenchymatous cells (figures 1, 2, and 3).

Testicle.—Of this tissue thirty-six cultures were made and in every case the extract caused marked stimulation of growth. Even after twenty-four hours the difference between the stimulated specimens and the controls was visible. In the case of the controls only a few branching cells were observed spreading from the edge of the tissue, but in the stimulated specimens these formed a well marked ring around the tissue. Growth rapidly progressed and by the fourth day the new tissue formed a mass four or five times the diameter of the original tissue (figure 7). Close to the cut edges the newly growing tissue

formed a solid mass indistinguishable from the original tissue, so that this appeared to have markedly increased in size. This is well shown in the figures of the stimulated and unstimulated specimens, the former of which is only half the magnification of the latter (figures 4 and 7). Outside the solid masses so formed the cells were widely spreading in all directions, so that a mosaic was formed apparently only one cell thick. Two types of cells were discernible: branching, irregular connective tissue cells, and more cuboidal cells with a dark staining nucleus and more distinct protoplasm. These latter cells were always grouped together to form tongue-shaped masses radiating from the sides of the tissue. In the controls the growth of the cells which were apparently parenchymatous in nature was much less marked, nearly all the cells being branching, elongated, and apparently of connective tissue, although even these cells were not growing to nearly so marked an extent as the similar type of cell in the stimulated specimens.

It is interesting to note that with this tissue there was no vacuolation of the plasma. If the cultures were left for a relatively long period without subculturing the cells became granular and degenerate, so that on staining only a mass of debris was seen, but in no case were large vacuoles formed such as are common with degenerate specimens of other tissues.

In the subcultures the connective type of cell rapidly overgrew the parenchymatous in the usual way, but the growth of the connective tissue type of cell was more marked and life could be prolonged to a greater extent in those cases where an extract had been used.

Liver.—Of this tissue thirty-six cultures were also made. In them the variation between the growth of parenchymatous and connective tissue cells is much more evident, for the parenchymatous cells of the liver form solid, deeply staining masses which can readily be distinguished from the connective tissue type. In all cases it was found that in the controls the characteristic feature was the presence of large club-shaped masses of cuboidal cells radiating from the cut edge into the plasma, while the connective tissue cells were present only in small numbers (figure 10). In the specimens to which the extract had been added there was a considerable in-

crease in the growth of the branching spindle cells, but only a few cuboidal cells were present. It is important to note that the parenchymatous cells were not only relatively but actually less in number in the tissues to which the extract had been added. The same characteristics were seen in subcultures which had been fixed in a relatively early stage. In the specimens with the extract there were well defined connective tissue overgrowth and only a few parenchymatous cells, whereas in the controls the amount of parenchymatous growth was extensive and the connective tissue growth less marked. Later, even in the specimens without extract, the cuboidal cells were overgrown by those of the connective tissue type.

Kidney.—Of this tissue fifty-four cultures were made. It was found necessary to exercise caution in drawing conclusions, for of all tissues this seems to be the one most likely to die, and in a series of cultivations several may die from no apparent cause. It was found, however, that in all cases where growth was present this was more marked in the stimulated specimens and that a large proportion of them showed growth. Not only was there an increase of parenchymatous growth, but the connective tissue cells were present also in large numbers. As I have previously shown, the growth of kidney tissue in normal plasma is characterized by the presence of cuboidal parenchymatous cells, very few connective tissue cells being present.

TABLE I.

Effect of Spleen Extract.

Tissue.	With extract.		Without extract.	
	Parenchymatous.	Connective.	Parenchymatous.	Connective.
Spleen...	Fair, slightly increased	Very good, considerably increased	Fair	Fair.
Thyroid..	Very good	Good	Fair	Moderate.
Testicle..	Very extensive	Extensive	Fair	Good.
Liver....	Slight	Good	Very good	Slight.
Kidney ..	Good	Good	Fair	Very slight.

Spleen extract would therefore seem always to stimulate the growth of connective tissue to a considerable extent. Its effect on the parenchymatous cells appears to differ with the tissues used. Of

the organs investigated, growth of these cells was markedly stimulated in the case of the testicle and thyroid, slightly stimulated in the kidney and spleen tissues, and inhibited in the case of the liver. These results are perhaps more clearly shown by table I.

MUSCLE EXTRACT.

In this investigation 259 cultures were made, the same tissues being used as with the spleen extract. As already mentioned, this extract had, owing to the toughness of the tissue, to be made by mincing the tissue with a pair of scissors. For this reason it was probably not so concentrated as were the other extracts, but here, as in other cases, controls were grown in plasma diluted with Ringer's fluid and it was found that the changes about to be described were not due to the fact that the plasma was diluted.

Spleen.—Seventy-eight cultures of this tissue were made. The effect of the extract was noticeable after growth had proceeded for a few hours. Thus after four hours the number of round cells which had emigrated into the plasma was in all cases more marked when the extract had been used. After twenty-four hours this difference was more evident, so that in the cultures with the extract the cells had wandered nearly to the limit of the plasma and formed a large mass around the tissue. The increased emigration of cells is liable to give rise to the belief that growth has been stimulated by the extract, but when care is taken to observe only the cuboidal parenchymatous cells this is seen not to be the case. In the preparations which have had the muscle extract added to them the growth of cuboidal cells is much less, and only a few of these cells are seen at the time when the same cells form a big area in the preparations without extract. On the other hand, the branching connective cells grow more rapidly in those cases in which the extract is added. In the case of the subcultures the preparations without extract show in the first subculture an overgrowth of the cuboidal parenchymatous cells, but those with the extract showed only radiating connective cells. In the third or fourth subculture the connective tissue cells are alone seen in both preparations. It would therefore appear that the muscle extract stimulates the growth of connective tissue cells but inhibits the parenchymatous cells of the spleen.

Thyroid.—Seventy-three cultures of this tissue were made. The results given were very definite. The specimens without the extract showed after two or three days the outgrowth of cuboidal cells which formed a mosaic around the tissue. The extent of the growth of these cells varied considerably with the animals used, but in all cases it was visible. Shortly after their appearance a few branching connective tissue cells were seen which soon grew out as radiating branches. In the case of the preparations with the extract the growth of the cuboidal cells was always much less, and in the majority of cases no such cells could be seen. The growth of the connective tissue cells was, however, much more rapid, so that by the fifth or sixth day a very wide network of branching cells was seen around the original tissue. The distinction between this wide branching network of connective tissue cells and the mosaic of cuboidal cells seen in the preparations without extract and in those treated with spleen extract was very clear.

Testicle.—Thirty-six cultures were made. The effect of this extract was not nearly so marked as that of the spleen. In all cases, however, there was a stimulation of the connective tissue type of cell. The branching cells wandered to a considerable distance into the plasma, forming an open network. The cuboidal parenchymatous cells also showed a distinct overgrowth in the stimulated specimens. In the unstimulated ones only small tongue-shaped processes of these cells were seen, whereas in the ones with the extract it was not uncommon to find a mosaic of these cells as large as the original piece of tissue. This overgrowth was observable both in the primary cultures and in the first subcultures. In the later subcultures these cells were overgrown in the usual way by the connective tissue cells, the growth of which was, as in the primary cultures, more marked in the stimulated specimens.

Liver.—Twenty-four cultures were made. In all cases the preparations containing the muscle extract showed more marked growth of the connective tissue type of cell, but again not to so great an extent as when the spleen extract was used. The effect upon the parenchymatous cells was not so definite. Those preparations with no extract again showed in all cases the characteristic masses of deeply staining cuboidal cells. In the preparations with the extract

these masses were also present, but they were never so extensive, the connective type of cell always preponderating. Similar changes were seen in subcultures. In the first subcultures both types of cell were seen, but the parenchymatous type of cell was more abundant in the specimens without the extract, the connective type in those with the extract. In later subcultures the connective tissue cell was more abundant in both.

Kidney.—Forty-eight cultures were made. As usual, a certain number of specimens died in both preparations. In cases in which growth took place, it was, however, clearly demonstrated that this was more marked in the specimens to which the extract had been added. Not only was the amount of growth more extensive, but in two parallel series those with the extract always showed a larger percentage of successful cultivations. As was to be expected from the results obtained with other tissues, the growth of connective tissue was more marked with the extract, but here again it was not nearly so extensive as when spleen extract had been used. In addition there was a very evident increased activity of the parenchymatous cells. As is usual with this tissue, these cells formed a definite mosaic apparently only one cell thick around the original piece of tissue, but, whereas in the controls these masses were small and projected only from certain points of the tissue, in the stimulated specimens fixed at a corresponding period of time they were much larger and often surrounded the tissue on all sides.

It would appear then that muscle extract stimulates the growth of connective cells whatever type of tissue be used, but this stimulation is not so marked as with spleen extract, although this difference may be due to the fact that the extract is weaker. On the other hand, some parenchymatous cells are stimulated and some are inhibited. It is important to note that the effects upon these parenchymatous cells differ from those of the spleen extract. Thus this type of cell is strongly stimulated in the case of the kidney, only slightly stimulated in the testicle, slightly inhibited in the liver, and more strongly inhibited in the thyroid and spleen. These results are shown in table II.

TABLE II.

Effect of Muscle Extract.

Tissue.	With extract.		Without extract.	
	Parenchymatous.	Connective.	Parenchymatous.	Connective.
Spleen ...	Slight or absent	Good, increased	Fair	Fair.
Thyroid..	Very slight	Good	Fair	Moderate.
Testicle..	Good, slightly increased	Good	Fair	Fair.
Liver....	Good	Good	Very good	Slight.
Kidney ..	Very good	Good	Fair	Very slight.

TESTICLE EXTRACT.

In investigating this extract 160 cultures were made, the same tissues being used as with the previous extracts. As the testicle of the adult rabbit is soft, an extract is readily prepared.

Spleen.—Thirty-six cultures were made. As in the case of the splenic and muscular extracts, the early emigration of cells was more marked than in the control specimens. The cuboidal cells of the parenchyma showed, however, no increase in any of the specimens. These cells were present, but, if anything, their growth was slightly less than that of the controls. In no case was the effect marked. On the other hand, the growth of connective tissue was considerably increased in all specimens. The extract was distinctly more active in this respect than the muscle extract, but was not so powerful as that of the spleen.

Thyroid.—Twenty cultures were made. In every case there was an increase in the growth of the parenchymatous cells, but not to so marked an extent as when the spleen extract was used. In all there was overgrowth of the connective tissue as compared with the controls. In the subcultures the connective tissue was also more marked when the extract was used, and more rapidly overgrew the parenchymatous cells.

Testicle.—Forty-eight cultures were made. As in the case of the other tissues, there was a considerable increase in the growth of the connective tissue, but never to so marked an extent as when the spleen extract was used. The growth of parenchymatous cells is also distinctly increased, these cells forming large mosaic-like areas when the corresponding controls showed small areas projecting

from the edge of the tissue (figure 8). Not uncommonly by the third day these masses of cuboidal cells formed in the stimulated specimens an area with a diameter as large as, or larger than, that of the original tissue. In no case, however, were these masses nearly so extensive as in the case of the preparations stimulated with splenic extract. In the subcultures the cuboidal cells were in the usual way rapidly overgrown by the connective tissue cells.

Liver.—Thirty-six cultures were made. In all cases there was some increase of the connective tissue cells, these cells being present at an earlier date and growing more rapidly. The parenchymatous cells were definitely decreased in numbers, there being only slight growth of small cuboidal masses from the edge of the tissue (figure 14). The same increased growth of the connective tissue was seen in the subcultures.

Kidney.—Twenty cultures were made. In all cases the tendency for vacuolation which is so marked with this tissue was more in evidence when the extract was used, and in such cases very little growth was seen. If, however, this vacuolation occurred early the cells might continue to grow in the plasma, and owing to the vacuole, were then completely separated from the original tissue. Under such circumstances no connective tissue cells were to be seen, it being probable that the cells are separated from the tissue before this type of cell has commenced to grow. If the growing portion remained attached to the original tissue there was always an increase in the numbers of connective tissue cells in those specimens with the extract. In all cases the growth of parenchymatous cells was much less in the specimens with the extract than in those without it.

Testicular extract appears, then, to stimulate the growth of connective tissue in all cases. This stimulation is more marked than is the case with muscle extract, but is not so extensive as when spleen extract is used. Certain of the parenchymatous cells are stimulated and some of them are inhibited. Thus the growth of this type of cell was always more marked in the case of the testicle and thyroid, was diminished in the case of the kidney and liver, and was but slightly if at all affected in the case of the spleen. These results are shown in table III.

TABLE III.

Effect of Testicle Extract.

Tissue.	With extract.		Without extract.	
	Parenchymatous.	Connective.	Parenchymatous.	Connective.
Spleen	Fair	Good	Fair	Fair.
Thyroid	Good	Good	Fair	Moderate.
Testicle	Very good	Good	Fair	Fair.
Liver	Very slight	Good	Very good	Slight.
Kidney	Slight	Good	Fair	Very slight.

THYROID EXTRACT.

In the investigation of this extract 148 cultures were made, the same tissues being again used. Owing to the small size of the thyroid gland, only small quantities of the extract could be prepared from each animal.

Spleen.—Sixteen cultures of this tissue were made. As with the other extracts, the specimens to which this extract was added showed an increase in the number of cells emigrating in the early stages. The growth of both parenchymatous and connective tissue cells was also definitely increased. By the third day the specimens with the extract already showed many branching connective tissue cells and masses of cuboidal parenchymatous cells, the corresponding cells being much less marked in the controls. By the fifth day the specimens with the extract were surrounded by large masses of parenchymatous cells, while close to the edge of the original tissue the connective cells formed a branching network difficult to distinguish from the tissue itself.

Thyroid.—Thirty-four cultures were made. The connective tissue cells were again increased, but not so extensively as when spleen extract was used, the amount of growth of this type of cell resembling that found when testicular extract was used. The parenchymatous cells were also somewhat more in evidence than in the controls, but the amount of stimulation of these cells was slight. The growth was not nearly so extensive as when splenic extract was used, and in fact in some cases it was but little more marked than in the controls.

Testicle.—Forty cultures were made. In all the experiments

there was a very marked increase in the growth of the parenchymatous cells. The growth of these cells was as extensive as with the use of splenic extract, so that by the fourth or fifth day large masses of these cuboidal cells were seen extending widely from the edge of the tissue into the surrounding medium. The connective tissue cells were, however, as in the case of the other tissues, not stimulated to nearly so marked an extent as by the spleen extract, although the growth was always more extensive than in the case of the controls. For this reason the masses of cuboidal cells were more clearly visible than when spleen extract was used (figure 6). In the subcultures the connective tissue grew more rapidly, so that the growth of the parenchymatous cells was not nearly so marked.

Liver.—Thirty-six cultures were made. The connective tissue growth was again increased to a moderate extent. The characteristic masses of deeply staining cuboidal cells were also more extensive with the use of the extract, the specimens in this particular differing markedly from those in which splenic extract was used (figure 12). The cuboidal cells formed large rounded or club-shaped masses growing irregularly from the edge of the tissue, but in some cases where the medium was thinner they formed mosaic-like masses apparently only one cell thick, which completely surrounded the original tissue and extended for some distance into the medium around. In the subcultures the connective tissue growth was more marked.

Kidney.—Twenty-two cultures were made. In these specimens again there was some increase in the connective tissue growth. The amount of growth of the parenchymatous cells was always less extensive than in the case of the controls. There was also a greater tendency for the liquefaction of the medium to take place, the original tissue being thereby separated by a fluid ring from the growing cells.

Thyroid extract appears, therefore, always to stimulate the growth of the connective tissue type of cell, whatever tissue is used, the amount of stimulation being about equal to that with testicular extract, but being less marked than that found with the use of splenic extract. Of the different tissues employed in this inves-

tigation the majority showed an increase in the growth of the parenchymatous cells when the extract was added. The extent of this stimulation differed, however, with the various cells. Thus, whereas this type of cell was markedly stimulated in the case of the spleen, testicle, and liver, it was only slightly increased in the case of the thyroid, and inhibited in the case of the kidney. These results are shown in table IV.

TABLE IV.

Effect of Thyroid Extract.

Tissue.	With extract.		Without extract.	
	Parenchymatous.	Connective.	Parenchymatous.	Connective.
Spleen	Very good	Good	Fair	Fair.
Thyroid	Good	Good	Fair	Moderate.
Testicle	Very good	Good	Fair	Fair.
Liver	Very good	Good	Very good	Slight.
Kidney	Slight	Good	Fair	Very slight.

In the second group of experiments one tissue only was cultivated from each animal. The extracts had been previously prepared from the tissues of another animal, as already described. The fluid was pipetted off the emulsified tissue and kept on ice in a sterile tube. In some cases it was used after twenty-four hours, but in other cases only after several days had elapsed. In this way it was possible to determine whether the effects of the extract were in any way altered by preserving it on ice. It will be seen that in all the experiments in this group the extracts were homogenous to the tissues, whereas in the last group they were autogenous. In no case was it evident that the homogenous extracts acted in any way differently from the autogenous extracts. The homogenous extracts were as efficient as the autogenous.

In this group 216 cultures were made.

Thyroid.—The thyroid was removed from a rabbit, and thirty-six preparations were made and cultivated in groups of six each in the following media: plasma, plasma and Ringer's fluid 2 to 1, plasma and thyroid extract 2 to 1, plasma and spleen extract 2 to 1, plasma and testicular extract 2 to 1, plasma and liver extract 2 to 1. All the extracts were one day old.

The results of these cultivations confirmed those made in the previous group. The controls showed a fair amount of growth of both the connective and parenchymatous cells, but with Ringer's fluid there was a slight increase in the growth of the connective tissue. The preparations with the thyroid and testicular extracts both showed a slight increase of the parenchymatous and connective tissue cells, while those with the spleen extract showed a considerable increase of both varieties of cells. Those with the liver extract showed, however, a marked diminution both in the parenchymatous and the connective tissue cells.

Testicle.—The testicle was removed and thirty-six preparations were made. The same culture media were used as in the last experiment, the extracts in this case being eleven days old. Here again the results confirmed those obtained in the first group of experiments. The control specimens showed a fair amount of parenchymatous and connective tissue growth (figure 4). With Ringer's fluid there was a definite increase in the amount of connective tissue growth although the parenchymatous cells were not increased (figure 5). With the thyroid extract there was a marked increase in the amount of parenchymatous growth and a slight increase in the connective tissue growth (figure 6), a similar result being obtained with the testicular extract (figure 8). With the splenic extract both the parenchymatous and connective tissue cells were greatly increased (figure 7). With the liver extract, as in the case with the thyroid tissue, there was no growth of the parenchymatous, and a greatly diminished growth of the connective tissue cells (figure 9).

A second experiment, performed on precisely the same lines, gave identical results, the extracts in this case being thirteen days old.

Kidney.—The kidney was removed and thirty-six preparations were made in groups of six specimens in each of the same culture media as before. The results agreed with those of the first group. In the controls there was good growth mainly of the parenchymatous cells, very few of the connective cells being present. In the specimens with Ringer's fluid the growth of connective cells was

rather more marked, but with this diluent the medium liquefied more readily and hence the growth of parenchymatous cells was less. With the thyroid extract the growth of parenchymatous cells was decreased as compared with the controls, but that of the connective cells was somewhat more marked. A similar result was obtained with the use of testicular extract. With the spleen extract there was a very definite increase in the amount of growth of both types of cell, but with the liver extract again there was very little or no growth.

Liver.—Thirty-six preparations were made in the same varieties of media, the extracts in this case being one day old. Good growth of both parenchymatous and connective tissue cells was obtained in the controls (figure 10). With Ringer's fluid the growth of the connective tissue cells was increased, but that of the parenchymatous cells was somewhat lessened (figure 11). With thyroid extract the connective growth was increased to a moderate extent, while the parenchymatous growth was considerably increased (figure 12). Testicular extract had a similar effect upon the connective cells, but diminished the growth of the parenchymatous cells (figure 14). This action is of interest, for in all the tissues previously considered these two extracts had a similar effect, but in the case of the liver tissue the one inhibits and the other stimulates the growth of the parenchymatous cells. With liver extract the growth of both types of cell was considerably diminished (figure 15). Splenic extract stimulates only the connective tissue growth (figure 13).

A second experiment was carried out on similar lines, but in this case the extracts were all twenty days old. It was found that in this case no growth occurred with any of the extracts, although good growth was seen in the preparations in simple plasma and in those in plasma and Ringer's fluid.

The results, therefore, of this series of experiments confirmed those of the first group, but in addition it was seen that liver extract apparently strongly inhibits the growth of both the connective tissue and parenchymatous cells of all tissues. The effects of other extracts are constant whether they are homogenous or autogenous

to the tissue used. Up to a certain period of time the extracts can be kept unchanged on ice, but after this time they appear to undergo a change.

CONCLUSIONS.

The cultivation of cells *in vitro* affords a valuable means of estimating the effects of tissue extracts.

Tissue extracts have a definite effect upon the growth of adult mammalian cells *in vitro*.

The majority of tissue extracts stimulate the growth of connective tissue, but liver extract inhibits it.

The extracts are to a certain extent specific in their action upon the growth of parenchymatous cells. Some cells are stimulated by one extract and inhibited by another, and those extracts which inhibit one type of parenchymatous cell may stimulate another type.

Homogenous and autogenous extracts are equally efficacious in their action upon the growth of cells.

The extracts may be preserved for a short period of time without suffering any change in their power of affecting the growth of cells.

EXPLANATION OF PLATES.[7]

PLATE 27.

Growth of adult rabbit thyroid in plasma and spleen extract.

FIG. 1. First culture. Fourth day.
FIG. 2. Subculture. Third day.
FIG. 3. Second subculture. Third day.

PLATE 28.

Growth of adult rabbit testicle in various media.

FIG. 4. Four days' growth in simple plasma.
FIG. 5. Four days' growth in plasma and Ringer's fluid.
FIG. 6. Four days' growth in plasma and thyroid extract.

PLATE 29.

Growth of adult rabbit testicle in various media.

FIG. 7. Four days' growth in plasma and splenic extract.
FIG. 8. Four days' growth in plasma and testicular extract.
FIG. 9. Four days' growth in plasma and liver extract.

[7] I am indebted to Mr. Summers for the photographs of the growing tissues.

PLATE 30.

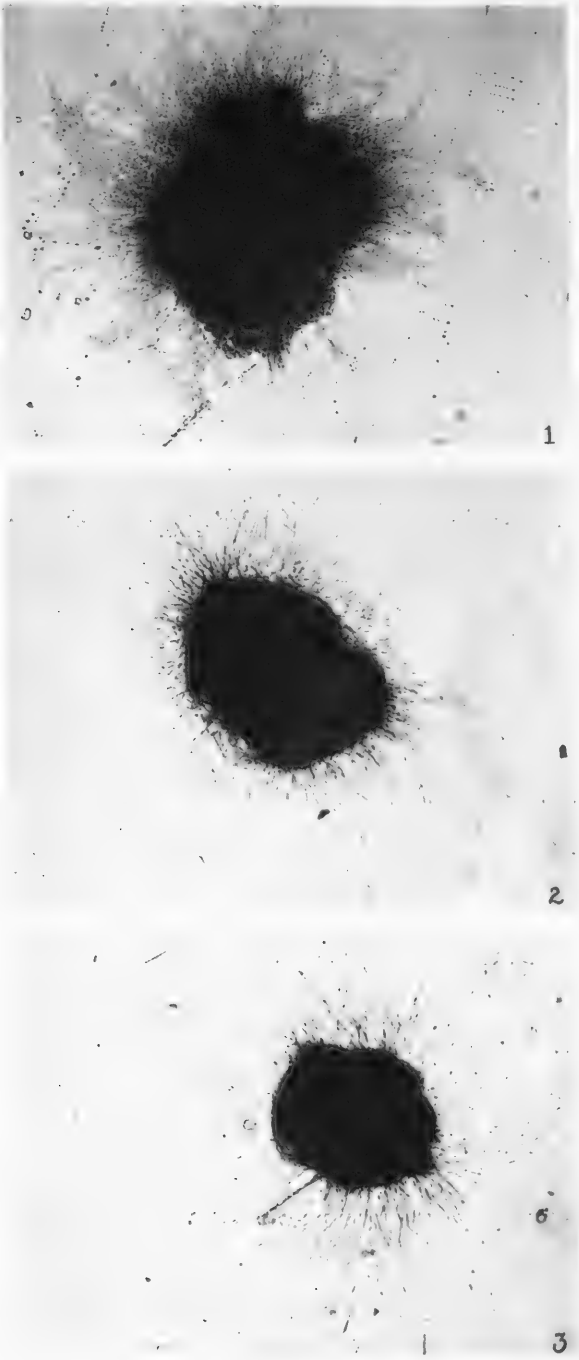
Growth of adult rabbit liver in various media.

FIG. 10. Four days' growth in simple plasma.
FIG. 11. Four days' growth in plasma and Ringer's fluid.
FIG. 12. Four days' growth in plasma and thyroid extract.

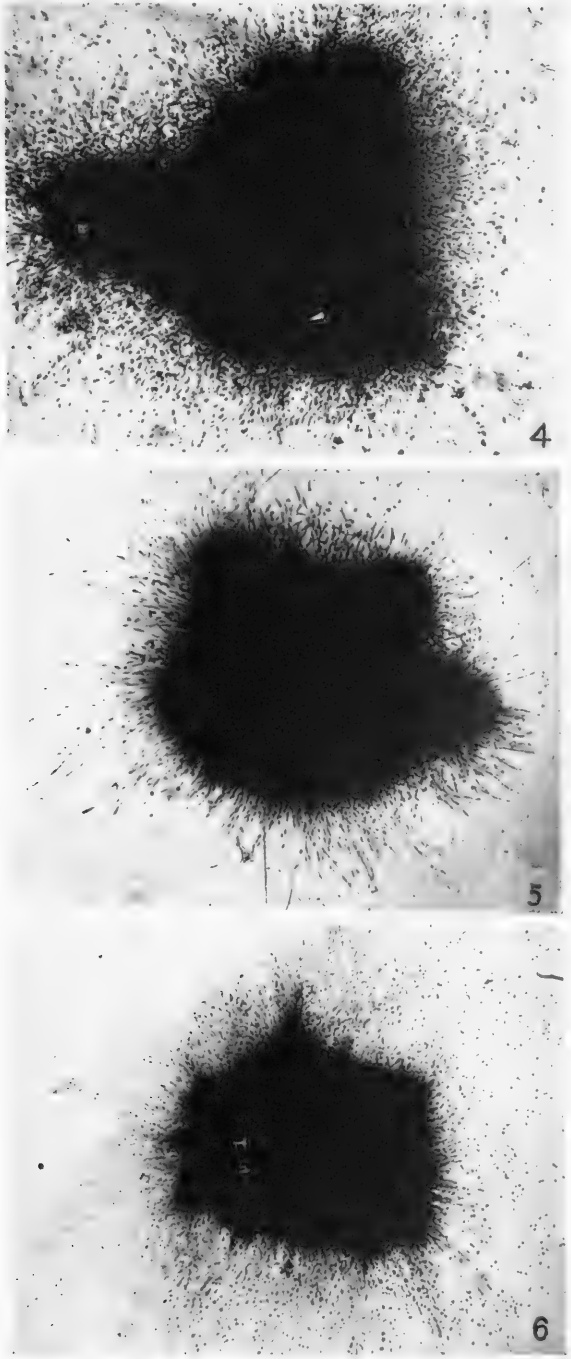
PLATE 31.

Growth of adult rabbit liver in various media.

FIG. 13. Four days' growth in plasma and splenic extract.
FIG. 14. Four days' growth in plasma and testicular extract.
FIG. 15. Four days' growth in plasma and liver extract.



(Walton: Growth of Adult Mammalian Cells *in Vitro*.)



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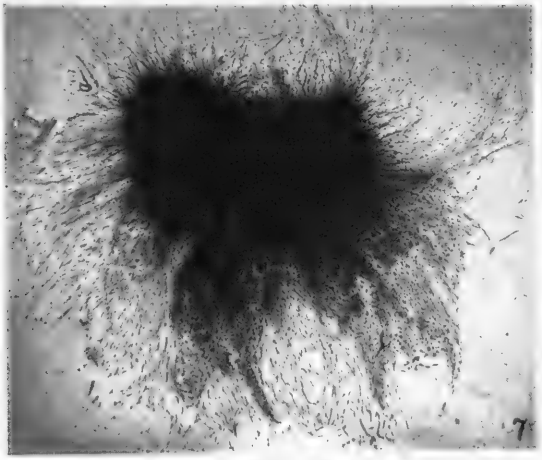

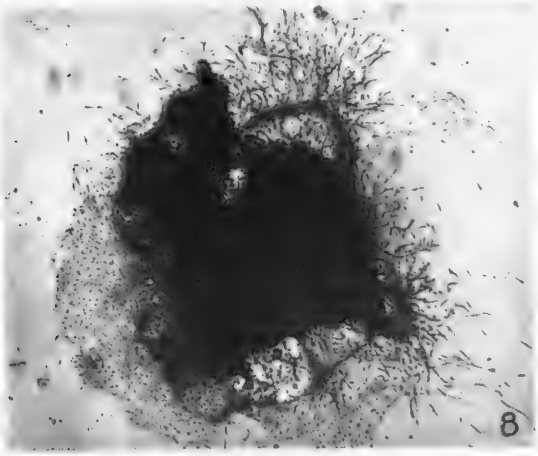

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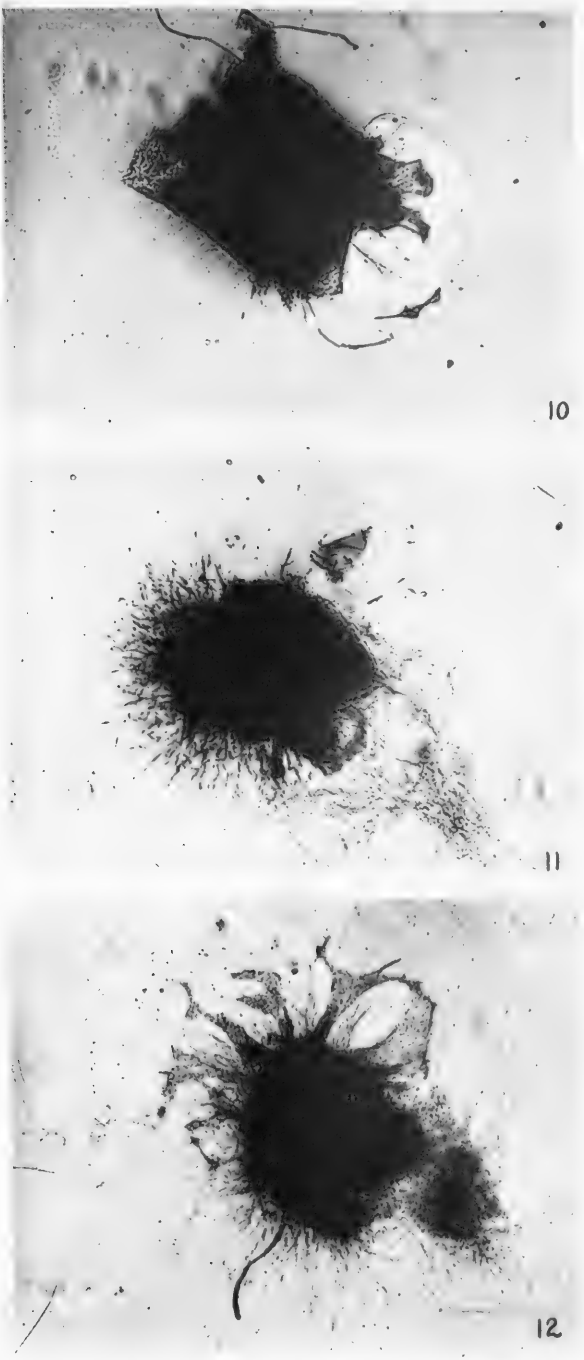

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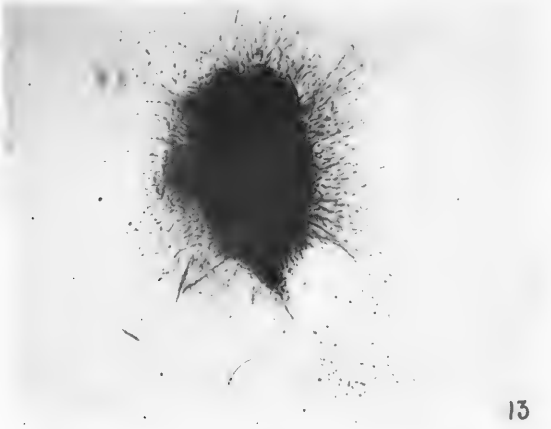

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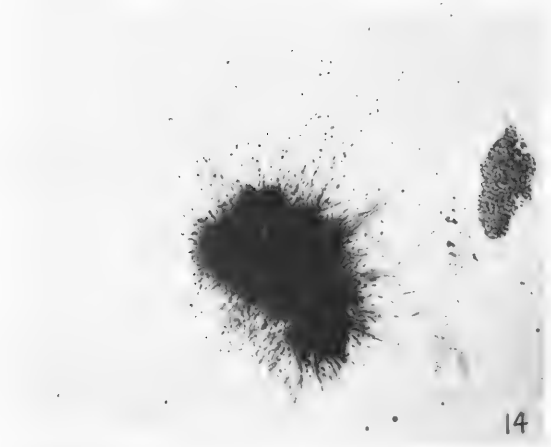

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(Walton: Growth of Adult Mammalian Cells *in Vitro*.)

OBSERVATIONS ON EXPERIMENTAL TYPHOID INFECTION OF THE GALL BLADDER IN THE RABBIT.*

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In recent years the question of immunity following antityphoid vaccination, the typhoid carrier problem, and the possibilities of chemotherapy have restimulated investigation of experimental typhoid infections in the lower animals.

A review of the present status of infection in animals below the apes is given by Hailer and Ungermann (1). In the case of the rabbit the original observation dates back to 1891, when Blachstein and Welch (2) noted that, after intravenous inoculation of colon and typhoid bacilli, rabbits may develop a lesion of the gall bladder which may persist for several months. Apparently no further work was done with this lesion at that time, but, in the last few years, infection of the organs of the rabbit and especially of the gall bladder have been used for various purposes by Doerr (3), Koch (4), Chiarolanza (5), Conradi (6), Hailer and Rimpau (7), Hailer and Ungermann (8), Hailer and Wolf (9), Uhlenhuth and Messerschmidt (10), Morgan (11), Johnston (12), Gay and Claypole (13), and Cummins and Cumming (14).

It has been shown that typhoid bacilli can be isolated from the organs for some time after injection, but that the gall bladder lesion is the most persistent source. After intravenous injection, the gall bladder apparently becomes infected by way of the blood stream as well as from the bile. It has also been shown that intravenous inoculation does not regularly result in producing gall bladder lesions and most of the experimenters in chemotherapy have given up this method for the direct inoculation of the gall bladder. The intravenous method of infection, however, more nearly resembles the natural method and has advantages for some purposes.

In this paper the following subjects are considered: (1) patho-

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genicity of a living sensitized vaccine, (2) pathogenicity of first transplant from a living sensitized vaccine, (3) the regular production of lesions, (4) the gall bladder lesion as a test of immunity, (5) the curative effects of vaccines, and (6) the practical bearing of experimental work. Altogether, ninety-seven animals have been used and forty gall bladder lesions have been observed.

PATHOGENICITY OF A LIVING SENSITIZED VACCINE.

The writer's attention was first attracted to the gall bladder lesion as a means of determining whether or not a living sensitized vaccine is pathogenic. As is well known, Metchnikoff and Besredka (15), as a result of their work with experimental typhoid in the chimpanzee, devised a living sensitized vaccine for protection. This vaccine has been criticized as likely to produce carriers, if not the disease itself. Metchnikoff and Besredka's own observations on the possibility of producing carriers in apes and man have been negative. My own experience with rabbits has been the same. Through the kindness of Prof. Besredka I secured some of his vaccine which was living at the time of use, as evidenced by the growth from the vaccine of a typical typhoid organism. The vaccine was given intravenously to nine animals, and subcutaneously to one animal, in doses of two to six cubic centimeters. Two animals were sacrificed on the day following intravenous injection, and cultures made from the bile were sterile. The other animals were examined after one month; cultures from the bile were negative in all cases for typhoid bacilli, and no macroscopic evidence of disease was found. It may, therefore, be concluded that intravenous injection of this vaccine does not produce gall bladder lesions in the rabbit, and by inference that no danger exists of producing carriers in man by subcutaneous injection.

PATHOGENICITY OF THE FIRST TRANSFER OF A LIVING SENSITIZED VACCINE.

While the original vaccine is apparently harmless, the first transplant on agar is pathogenic, as is indicated by the following experiment.

Nov. 18, 1913. A large agar slant was inoculated with vaccine and, after twenty-four hours, the growth was collected in 20 c.c. of salt solution and 6 c.c. were given intravenously to a rabbit weighing 3,000 gm. On Dec. 23, the animal was killed and a well marked lesion of the gall bladder was found and the bile contained a pure culture of typhoid bacilli.

Hence while the vaccine itself is apparently safe, it has possibilities of harm in case it became accidentally deposited on media suitable for multiplication. This is a point of some practical importance in the military service in which ampules may unavoidably be broken in transportation or in camp. According to the results of this experiment, any water or food accidentally contaminated with this vaccine might transmit the disease to non-immunes. It may, therefore, be stated that a living sensitized vaccine is not entirely safe to handle, as is a killed vaccine (16).

ATTEMPTS TO PRODUCE GALL BLADDER LESIONS REGULARLY BY INTRAVENOUS INJECTION.

While the above work was in progress, Gay and Claypole (13) published a paper in which the authors stated that regular infections had been produced by intravenous injection of growths from rabbit blood agar. In order to try the effect of this procedure, the strain from the living vaccine was carried on rabbit blood agar and passed through lesions in five animals, as is seen in table I. The growth on a blood agar slant was suspended in twenty cubic centimeters of salt solution, and two cubic centimeters, or one half the fatal dose, were given intravenously and the animals were examined after one month.

TABLE I.

Attempts to Produce Gall Bladder Lesions with Transfers from Besredka's Vaccine on Blood Agar.

No. of passage.	No. of animals.	Positive.	Negative.
1	1	1	0
2	6	1	5
3	3	1	2
4	1	1	0
5	5	1	4
	16	5 (31%)	11

It will be seen that with this strain cultivation on blood agar had no effect in maintaining pathogenicity, nor did passage through four animals in series. In the fifth transfer only one animal out of five became infected. In this connection it should be noted that the strain Gay and Claypole worked with was recently isolated.

In order to determine the effect of the age of the culture several strains were tried with the following results. One half the fatal dose of an agar or bouillon growth was given intravenously and the animals were examined after one month (table II).

TABLE II.

Attempts to Produce Gall Bladder Lesions with Various Strains Given Intravenously.

Strain.	Age of strain.	No. of animals.	Positive.	Negative.
Clark	1 mo.	8	6	2
Fuguet	1 mo.	2	1	1
Andrews	1 mo.	4	0	4
Clark	3 mos.	4	0	4
Rawlings	14 yrs.	6	0	6
Dorset	15 yrs.	5	1	4
Besredka	?	16	5	11
		45	13 (28.8%)	32

The conclusion to be drawn from these experiments is that freshly isolated strains produce lesions in a higher percentage than old strains, but even freshly isolated strains cannot always be depended on to produce lesions by the intravenous method. This conclusion agrees with that of nearly every investigator and has been emphasized by Uhlenhuth and Messerschmidt, and Hailer and Ungermann. As stated above, these authors have given up the intravenous method for the more certain method of direct inoculation of the gall bladder.

THE GALL BLADDER LESION AS A TEST OF IMMUNITY.

Gay and Claypole also state that the gall bladder lesion can be used as a test of immunity conferred by vaccines. Evidently such a test in an animal would be of great value in many ways, but unfortunately this lesion does not seem to be suitable for such a

purpose. The instantaneous introduction of a large number of bacilli directly into the circulation seems to break down any artificial immunity produced by vaccines. Uhlenhuth and Messerschmidt (10) have been unable to protect animals from infection with the Pfeiffer-Kolle vaccine, although immune bodies were present in large quantities. The infecting dose was given directly into the gall bladder. My experience has been the same with the Army vaccine, using the intravenous route. Although it is impossible to carry on well controlled experiments with the intravenous method when the results are so uncertain, the positive results have some significance.

Ten rabbits were used. Six were vaccinated with 100 million subcutaneously, followed at weekly intervals by two doses of 200 million, intravenously. Four were vaccinated similarly with doses of 500 and 1,000 million. Ten days after the last vaccination, all were given an intravenous injection of about one half the fatal dose of Besredka's organism. After one month, two animals of each set showed definite infections of the gall bladder. The percentage of infections was 40, while the percentage of infections among controls (16) was 31.

Hence no protective effect of vaccination could be demonstrated in the rabbit. As the protective effect of the same vaccine in man is well known, this result simply means that the conditions of the experiment in the rabbit are not comparable with those in natural infections in man.

ATTEMPTS TO CURE THE GALL BLADDER LESION BY VACCINES.

Johnston (12) states that vaccines may have some curative effect on the lesion of the gall bladder. Uhlenhuth and Messerschmidt (10) were unable to obtain such a result, and Cummins and Cumming (14) also state that vaccine treatment did not influence the finding of living bacilli in the liver and gall bladder. I have also been unable to demonstrate any curative effect of vaccines.

Two rabbits were given 1 c.c. of a broth culture of Clark strain intravenously on Apr. 27, 1914. Plating of feces was positive to typhoid bacilli on May 15, 1914. Both animals were then given subcutaneously four doses of vaccine, one dose every five days, running from 10 to 80 million. One week after the last dose the animals were autopsied, and the bile in both cases yielded a pure culture of typhoid bacilli.

Two human carriers who have been studied in this laboratory and reported by Leary (17) were treated over several months with autogenous and stock vaccines and failed to show any marked diminution in the excretion of bacteria. Both cases were operated on and the gall bladders, containing pure cultures of the organism, were removed. Excretion of typhoid bacilli in the stools ceased permanently in nine days. It is, of course, not claimed that removal of the gall bladder will cure all carriers, because we know that the bile passages and intestinal mucosa may be affected in some cases. But our experience with the curative effect of vaccines has not been encouraging either clinically or experimentally.

PRACTICAL BEARING OF EXPERIMENTAL WORK.

In this laboratory our chief interest in experimental infections has naturally centered in their possible relation to immunity following vaccination. As is well known, the present army vaccine was devised in 1908 by Major F. F. Russell of the Medical Corps, on the basis of German and English experience. During the last five years we have manufactured over 1,700,000 cubic centimeters for the Army, Navy, National Guard, and other departments of the Government, and over 250,000 men have been immunized. The results of the use of this product have been fully detailed by Major Russell (18) in a number of contributions to our knowledge of this subject. It is only necessary here to emphasize the fact that the conditions of its use have been those of extensive experimentation. The subjects are exposed to infection in many parts of the world and are under close observation for several years at least. The results have exceeded all expectations, and, on the basis of English and American experience, we can now give a definite answer to some of the problems of immunity in man.

In the first place it is clear that the conclusions reached by Metchnikoff and Besredka from their work on chimpanzees cannot be accepted for man. These authors found that a whole killed vaccine did not protect their animals. But they used tremendous infecting doses,—the contents of a whole Kolle flask. On the other hand, they were able to protect with a living sensitized vaccine. But, as

has already been pointed out, this vaccine is not entirely safe to handle and its use could certainly not be made compulsory. Hence, if we followed these experimental results strictly, we would not be using any vaccine and the splendid results would have been lost. Inasmuch as the final object of all experimental work on this subject is the protection of man, it would be a *reductio ad absurdum* to give up the goal, once reached, because we cannot duplicate the conditions in animals.

In the same way the gall bladder lesion in the rabbit fails as an index of immunity from vaccination in relation to man. For an attack on the typhoid carrier problem this lesion seems ideal, and it may possibly serve in advancing the chemotherapy of typhoid fever. It certainly gives an admirable experimental opportunity for testing methods of isolating typhoid bacilli from the feces. But the claim of Gay and Claypole, that this lesion can be used as a test of immunity from vaccination, seems unfounded, and for the same reason as in the case of the chimpanzees. Human conditions cannot be duplicated and immunization which is known to protect man is not effective under these different conditions.

While the subject was developing, many products were proposed; the whole killed vaccine, autolysates, residues, fractions, etc. When it became evident that a whole killed vaccine had some advantages, other questions arose. What strain should be used? Should it be virulent or avirulent? Should it be fresh or old? Should several strains be used? Should they be sensitized? A great deal of work done with immune bodies has given no convincing answer to these questions. As a result of actual trial we can say that a whole vaccine killed by heat at 53° C. and preserved with 0.25 per cent. tricresol protects human beings from typhoid fever; that an old avirulent strain is effective; that a single strain is effective against typhoid in all parts of the world (19); that doses of 500 and 1,000 million at intervals of seven or ten days are effective; that two doses protect for two years and that three doses protect for a longer period not yet exactly determined; that the vaccine keeps its immunizing properties for at least four months.

Gay and Claypole say that "Empiricism rather than experimentation has largely determined the method employed." On the con-

trary, experimentation has marked every step of the evolution of our present vaccine since the first paper on the subject in 1896, by Pfeiffer and Kolle (20). The only peculiarity has been that the teachings of clinical experience have been given some weight; and that experimental results have been taken for just what they were worth and no more. No claim is made that the Army vaccine is the *summum bonum* in antityphoid vaccines. Improvements may come, but before our results are improved upon, they must be equalled. And they must be equalled, not in a few chimpanzees or rabbits, but in large numbers of human beings under observation for several years.

At present the subjects which give concern are, naturally, the length of immunity and the keeping qualities of the vaccine. Evidence on these points is accumulating from actual experience, and we know of no other safe method of reaching conclusions on these points.

SUMMARY.

1. Besredka's living sensitized vaccine, given intravenously, does not produce a typhoid lesion of the gall bladder in the rabbit.

2. The first transplant of this vaccine is capable of producing this lesion. Hence this vaccine is not entirely safe to handle.

3. Regular infections of the gall bladder have not been produced by carrying a known pathogenic strain on rabbit blood agar, by successive passage through animals, or by the use of freshly isolated strains.

4. No evidence could be demonstrated in the rabbit of the immunity produced in man by vaccination with a whole killed vaccine.

5. Vaccine treatment did not cure the gall bladder lesion.

6. With the present methods of producing infections in the chimpanzee and the rabbit, neither of these animals is suitable for deciding the problems of the immunization of man by vaccines. These problems must be settled, as some of them already have been settled, by actual experience with large numbers of men kept under close observation.

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PROTEOTOXINS (ANAPHYLATOXINS) AND VIRULENCE.*

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I.

In a series of experiments reported in another paper (1), the writers have shown that guinea pigs, previously injected with sublethal doses of bacterial proteotoxin (anaphylatoxin), may acquire slightly but definitely increased tolerance to these poisons. In the course of this work the material used for injection consisted of the supernatant fluid remaining after vigorous and prolonged centrifugation of mixtures of typhoid bacilli and fresh guinea pig serum. Although centrifugation was sometimes continued for two hours and more, the process never entirely freed the serum of bacteria, in spite of the fact that a settling of the microörganisms was often facilitated by the presence of normal agglutinins in the guinea pig serum, and, occasionally, by the addition of small amounts of inactivated, strongly agglutinating antityphoid serum.

The filtration of the preparation through Berkefeld candles freed it of bacteria, but, as reported in a previous paper, also entirely removed the toxic properties, and for this reason such a procedure could not be utilized.

The fluids obtained were always perfectly clear and showed no cloud on shaking, but on cultural examination, in which several loopfuls were planted, a few colonies always appeared. The majority of the animals injected with these fluids, if they survived acute shock, went on to complete recovery. Not infrequently, however, a guinea pig that had recovered entirely from the immediate effects of the poison would die, in the course of two or three days, of typhoid infection, cultures from the heart's blood showing typhoid bacilli abundantly present.

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Since the minimal fatal dose of our typhoid culture for guinea pigs of about 200 grams was one tenth to one fifteenth of an agar slant, and it was plain that nothing like this amount could have been injected with the two or three cubic centimeters of proteotoxin (anaphylatoxin) in the animals described above, it seemed as though the poison itself might have possessed aggressin-like properties by virtue of which the sublethal amounts of bacilli, remaining in the serum after centrifugation, had been rendered more powerfully pathogenic.

Our first experiments were aimed purely at the determination of this point. The procedure in these preliminary experiments consisted in determining the minimal lethal dose of the bacteria in question for guinea pigs of a certain weight, and then injecting smaller quantities of bacteria together with sublethal doses of the proteotoxin (anaphylatoxin).

The proteotoxin was prepared throughout with typhoid bacilli, and the bacilli, in proportions of one half agar slant to four cubic centimeters, were digested for five or six hours at 37.5° C. with fresh normal guinea pig serum. Centrifugation was then carried on for one to two hours, cultures were taken, and this fluid was added to the quantities of bacteria measured for the experiment.

EXPERIMENT A.

Typhoid Bacilli Injected Intraperitoneally with Proteotoxin (Anaphylatoxin).

No. of animal.	Weight.	Dose, agar slant.	Proteotoxin (anaphylatoxin).	Salt solution.	Result.
1	200 gm.	1/100	3 c.c.	——	Died in 22 hrs.
2	215 gm.	1/100	3 c.c.	——	Died in 18 hrs.
3	210 gm.	1/200	3 c.c.	——	Died in 18 hrs.
4	215 gm.	1/200	3 c.c.	——	Died in 35 hrs.
5	215 gm.	1/300	3 c.c.	——	Remained alive.
6	215 gm.	1/10	——	3 c.c.	Died in 52 hrs.
7	205 gm.	1/20	——	3 c.c.	Died in 18 hrs.
8	225 gm.	1/50	——	3 c.c.	Remained alive.

It is here seen that 1/200 c.c. of an agar slant was fatal if injected with 3 c.c. of the proteotoxin, whereas with salt solution alone 1/20 was the minimal lethal dose. 3 c.c. of the anaphylatoxin alone did not kill. The same result is shown in the following protocol.

EXPERIMENT B.

Typhoid Bacilli Injected Intraperitoneally with Proteotoxin (Anaphylatoxin)

No. of animal.	Weight.	Dose, agar slant.	Proteotoxin (anaphylatoxin).	Salt solution.	Result.
1	225 gm.	1/100	2.5 c.c.	——	Died in 3 dys.
2	220 gm.	1/200	2.5 c.c.	——	Died in 4 dys.
3	230 gm.	1/10	——	2.5 c.c.	Sick, recovered; remained alive.
4	235 gm.	1/20	——	2.5 c.c.	Remained alive.
5	230 gm.	——	3.0 c.c.	——	Not sick; remained alive.

These experiments show that, when injected intraperitoneally, the proteotoxin enhances the virulence of the typhoid bacilli. It should be noted that, in the case of typhoid infection, these protocols represent unusually favorable results. Although distinct differences pointing in the same direction were noticeable in all the experiments, these two represent the best results obtained. More regular results were obtained when the injections were made intravenously.

EXPERIMENT C.

Typhoid Bacilli Injected Intravenously with Proteotoxin (Anaphylatoxin) Produced with Typhoid Bacilli. 3.5 Cubic Centimeters of the Anaphylatoxin Used Killed a Guinea Pig Weighing 240 Grams in Three Minutes with Acute Shock.

No. of animal.	Dose.	Proteotoxin (anaphylatoxin).	Salt solution.	Result.
1	1/40	1.5 c.c.	——	Very sick, but recovered.
2	1/100	1.5 c.c.	——	Died in 48 hrs.; typhoid bacilli in heart's blood.
3	1/200	1.5 c.c.	——	Sick, but recovered.
4	1/10	——	1.5 c.c.	Sick, but recovered.
5	1/20	——	1.5 c.c.	Remained well.
6	1/40	——	1.5 c.c.	Remained well.
7	——	2.0 c.c.	——	Remained well.

This experiment again illustrates the aggressive effect of the poison, but here as in several other experiments irregularities occurred in that the largest dose given with the poison failed to kill, while a smaller dose was fatal (guinea pigs 1 and 2). We cannot explain this and can attribute it at present only to individual differences in the animals, although it occurred a number of times.

In all the preceding cases the proteotoxin was produced with typhoid bacilli, and typhoid bacilli were used in the experiment. The question naturally arose as to whether this aggressive action of the proteotoxin was specific or whether proteotoxin produced

with typhoid bacilli would show the same action when injected with other bacteria. This was a natural question, especially since all recent work has seemed to indicate that the proteotoxins were functionally alike whatever the method used for their production. For this reason the following experiments were carried out in which proteotoxin produced with typhoid bacilli was combined with staphylococci, and in one case with prodigiosus bacilli.

EXPERIMENT D.

Staphylococci Injected Intraperitoneally with Proteotoxin (Anaphylatoxin) Produced with Typhoid Bacilli.

No. of animal.	Dose, agar slant.	Proteotoxin (anaphylatoxin).	Salt solution.	Result.
1	1/100	2 c.c.	——	Died in less than 18 hrs.
2	1/200	2 c.c.	——	Died in less than 18 hrs.
3	1/400	2 c.c.	——	Died in 20 hrs.
4	1/400	2 c.c.	——	Died in less than 18 hrs.
5	1/10	——	2 c.c.	Remained alive.
6	1/50	——	2 c.c.	Remained alive.
7	1/100	——	2 c.c.	Remained alive.
8	——	3 c.c.	——	Died in 5 dys. with typhoid bacilli in heart's blood.

Staphylococci were found in the heart's blood of guinea pigs 2 and 3, the only ones of the series that were autopsied.

EXPERIMENT E.

Staphylococci Injected Intraperitoneally with Proteotoxin (Anaphylatoxin) Produced with Typhoid Bacilli.

No. of animal.	Weight.	Dose, agar slant.	Proteotoxin (anaphylatoxin).	Salt solution.	Result.
1	250 gm.	1/100	3 c.c.	——	Died in 18 hrs.
2	230 gm.	1/100	3 c.c.	——	Died in 16 hrs.
3	250 gm.	1/200	3 c.c.	——	Died in 22 hrs.
4	225 gm.	1/200	3 c.c.	——	Died in 21 hrs.
5	250 gm.	1/400	3 c.c.	——	Died in 18 hrs.
6	215 gm.	1/800	3 c.c.	——	Died in 29 hrs.
7	175 gm.	1/2	——	3 c.c.	Died in 19 hrs.
8	220 gm.	1/5	——	3 c.c.	Died in 14 hrs.
9	210 gm.	1/10	——	3 c.c.	Remained alive.
10	235 gm.	1/20	——	3 c.c.	Remained alive.
11	240 gm.	——	3 c.c.	——	Died in 3 dys.

Autopsies on guinea pigs 5 and 6 showed pure cultures of staphylococci in the heart's blood. No. 11 showed typhoid bacilli in the heart's blood.

EXPERIMENT F.

Prodigiosus Bacilli Injected Intraperitoneally with Proteotoxin (Anaphylatoxin) Produced with Typhoid Bacilli.

No. of animal.	Dose, agar slant.	Proteotoxin (anaphylatoxin).	Salt solution.	Result.
1	1/50	2 c.c.	——	Died in 18 hrs.
2	1/100	2 c.c.	——	Died in 18 hrs.
3	1/100	2 c.c.	——	Died in 18 hrs.
4	1/200	2 c.c.	——	Died in 18 hrs.
5	1/200	2 c.c.	——	Died in 20 hrs.
6	1/10	——	2 c.c.	Remained alive.
7	1/20	——	2 c.c.	Remained alive.
8	1/40	——	2 c.c.	Remained alive.
9	1/80	——	2 c.c.	Remained alive.
10	——	3 c.c.	——	Died in 5 dys.

Prodigiosus bacilli were found in the heart's blood of guinea pig 1, the only one of this series autopsied for this purpose. In guinea pig 10, the proteotoxin control, death ensued in five days, from generalized typhoid infection due to the few organisms remaining in the fluid after centrifugation. Without invalidating the present series, therefore, this forms an additional corroboration of the point made in experiments A and B.

EXPERIMENT G.

Staphylococci Injected Intravenously with Proteotoxin (Anaphylatoxin) Produced with Typhoid Bacilli.

No. of animal.	Weight.	Dose, agar slant.	Proteotoxin (anaphylatoxin).	Salt solution.	Result.
1	125 gm.	1/40	1 c.c.	——	Died in 2 dys.
2	185 gm.	1/80	1 c.c.	——	Died in 2 dys.
3	160 gm.	1/10	——	1 c.c.	Not sick.
4	190 gm.	——	2 c.c.	——	Slight shock, but recovered and remained alive.

Blood cultures taken from the heart's blood of guinea pigs 1 and 2 just before and after death showed staphylococci in pure culture.

EXPERIMENT H.

Staphylococci Injected Intravenously with Proteotoxin (Anaphylatoxin) Produced with Typhoid Bacilli.

No. of animal.	Weight.	Dose, agar slant.	Proteotoxin (anaphylatoxin).	Salt solution.	Result.
1	185 gm.	1/20	1 c.c.	——	Died in 48 hrs.
2	195 gm.	1/50	1 c.c.	——	Died in 48 hrs.
3	180 gm.	1/10	——	1 c.c.	Remained alive.
4	175 gm.	——	2 c.c.	——	Slight shock, but recovered and remained alive.

Staphylococci and typhoid bacilli, the latter in relatively small number, were recovered in blood plates made of the heart's blood of guinea pigs 1 and 2. The fact that the proteotoxin control, guinea pig 4, was able to overcome completely the typhoid bacilli present in twice the amount of proteotoxin indicates that the staphylococci in this case conversely aided the typhoid infection. The significance of this fact in connection with mixed infections in man will be discussed in our conclusions.

It is noticeable throughout these experiments that, although distinct enhancement of the virulence of typhoid bacilli is accomplished by the addition of the proteotoxin, the results are much sharper and more convincing when staphylococci are the infecting agents. We are inclined to attribute this to the fact that proteotoxin is much more easily formed by the aid of typhoid bacilli than with staphylococci. In the former, therefore, the mere injection of the bacilli leads to the formation of moderate amounts of the poison, making the difference between experiment and controls a purely quantitative one, whereas in the case of the cocci, no proteotoxin, or practically none, is formed when the bacteria alone are injected. The difference between experiment and control here, therefore, shows much more sharply the effect of the proteotoxin.

In the above experiments we have attributed the increased virulence of the bacteria, when injected together with proteotoxin, to an aggressive property possessed by the poison. This is obviously the only possible interpretation in the case of the staphylococcus and prodigiosus experiments in which the bacteria used in the production of the proteotoxin were of a species other than those later added to the poison and leading to death. In the first experiments, however, in which typhoid bacilli were used, both in the poison production and later in the experiment, another possible contributing factor must be considered. In our earliest accidental observations on the fact that animals would often die of typhoid infection after receiving only the minute doses represented by the bacteria not removable by centrifugation, it had occurred to us that these microorganisms might have been enhanced in virulence because of contact with the serum during the period of incubation incidental to proteotoxin production. This is the more obvious in the light of the recent discovery by Jobling and Petersen (2) that, during such

contact, bacteria will absorb anti-enzymes out of the serum. It might also be correlated with the observation, made by us in isolated cases, that the bacteria cultivated directly from the dead animal showed demonstrably increased resistance to agglutination. However, this latter point is being subjected to further investigation.

Accordingly we performed experiments to determine whether or not typhoid bacilli would increase in virulence after exposure to contact with fresh guinea pig serum, as in the production of the poison.

The procedure adopted consisted in preparing an emulsion of typhoid bacilli, dividing it into two parts, adding definite volumes of fresh guinea pig serum to one, and equivalent volumes of salt solution to the other. After exposure of from five to six hours, both portions were centrifugalized until the supernatant fluids were clear, and approximately quantitative, comparative determinations of virulence could be made with the sediments. The experiments were necessarily inexact owing, in the first place, to the bactericidal action of the fresh guinea pig serum and, in the second, to differences in sedimentation during centrifugation of the bacteria in the salt solution and in the serum. Both errors would tend to favor a negative answer to our query. Careful and repeated performance of this experiment, however, has satisfied us that typhoid bacilli exposed to fresh normal guinea pig serum for five to six hours at 37.5° C. do not gain in virulence sufficiently to influence the results of our preceding experiments. This is as far as we have gone in this direction for the present, since further elaboration of the problem was unnecessary for the specific purposes of the work before us.

Our experiments then have thus far shown that sublethal doses of typhoid bacilli, staphylococci, and, in one case, prodigiosus bacilli could be rendered lethal, if proteotoxin (anaphylatoxin) was administered with them. This aggressive action on the part of the proteotoxin was shown to be non-specific, in that when produced with typhoid bacilli, the poison would serve to augment the infectious properties of staphylococci as well as, or even more effectively than it did those of the typhoid bacilli themselves. This effect of the proteotoxin, however, was not noticeable unless considerable

quantities were employed, no results being obtained unless from 1 tc 1.5 cubic centimeters, representing one fourth to one half of a minimal lethal dose, were injected.

II.

It now became of interest to determine, if possible, the mechanism upon which this aggressive action of the proteotoxin depended. It seemed logical first to investigate whether the proteotoxin acted anti-opsonically; that is, whether it hindered the phagocytosis of the bacteria in the presence of normal serum. Accordingly experiments like the following were carried out.

The anaphylatoxin was prepared from typhoid bacilli, as in the other experiments. That used in the following protocol produced severe shock but not death in a guinea pig weighing 160 grams, when 3 c.c. were injected intravenously.

The bacteria employed were staphylococci emulsified in salt solution from a twenty-four-hour agar slant.

The leucocytes used were obtained by aleuronat injection into the peritoneum of a guinea pig. They were washed once only, since complete removal of the serum seemed unnecessary for this experiment.

The following tubes were prepared, incubated for forty minutes, and slides were made for the determination of phagocytosis in the usual manner.

EXPERIMENT I.

1.	Fresh guinea pig serum	0.2 c.c.
	Leucocytic emulsion	0.3 c.c.
	Staphylococcus emulsion	0.2 c.c.
	Salt solution	0.8 c.c.
	Phagocytic index = 20 +	
2.	Fresh guinea pig serum	0.2 c.c.
	Leucocytic emulsion	0.3 c.c.
	Staphylococcus emulsion	0.2 c.c.
	Proteotoxin emulsion	0.8 c.c.
	Phagocytic index = 20 +	
3.	Leucocytic emulsion	0.3 c.c.
	Staphylococcus emulsion	0.2 c.c.
	Salt solution	1.0 c.c.
	Phagocytic index = 2.1	
4.	Leucocytic emulsion	0.3 c.c.
	Staphylococcus emulsion	0.2 c.c.
	Proteotoxin	1.0 c.c.
	Phagocytic index = 12	

It is apparent from this experiment both in tubes 2 and 4 that the presence of the proteotoxin did not hinder opsonic action. Al-

though the staphylococcus opsonins were considerably diminished by absorption with typhoid bacilli it is evident in tube 4, even after subtracting the opsonic action due to traces of serum carried in with the leucocytes, far from hindering phagocytic action, the proteotoxin still carries with it opsonin for the staphylococci, a fact surprising in itself when we consider the absorption of the serum with not inconsiderable quantities of typhoid bacilli and its exposure, during proteotoxin production, to 37.5° C. for five hours.

It is also possible that, although the heat-sensitive opsonic substances may have been completely or almost completely removed by the exposure to 37.5° C. for five hours, the high phagocytic index in tube 4 may be explained by reactivation of a heat-stabile sensitizing element by the small amount of fresh serum adherent to the leucocytes, which were not thoroughly washed in these experiments. We did not further investigate this purely incidental point.

We shall not cite other experiments since those similarly performed all point, in agreement with this one, to the fact that the proteotoxin, as produced by us, does not exert anti-opsonic action.

From the fact thus demonstrated, that the proteotoxin does not hinder the phagocytosis of staphylococci in the presence of normal active serum, we may reasonably conclude that its action is not anti-opsonic. It is also apparent from the preceding protocol that, although there is slight cytotoxic action on the phagocytic cells, a fact which other experiments lead us to suspect, this is not in itself sufficient to be held responsible for the virulence-enhancing influence of the proteotoxin. For the leucocytes still actively ingested the bacteria in spite of contact, for thirty minutes and longer, with proteotoxin in concentration relatively far greater than any that would be likely to occur in the circulation of the infected animal.

These possibilities being excluded, we turned our attention to the leucopenia which is known to ensue upon the injection of poisons of this kind into animals. The experiment that follows is one of several planned to throw light on this point.

EXPERIMENT II.

After it had been determined on a number of animals that a suitably adjusted quantity of proteotoxin, injected into a guinea pig, regularly produces a fall in the total number of leucocytes, varying in degree, speed of onset, and duration

with the severity of the poisoning, two guinea pigs were intravenously injected as follows:

1. Weight 250 gm.
   Staphylococci $\frac{1}{20}$ agar slant.
   Proteotoxin, 2 c.c.
2. Weight 260 gm.
   Staphylococci $\frac{1}{20}$ agar slant.
   Salt solution 2 c.c.

The leucocyte counts of these animals were as follows:

	1.	2.
3 counts during a period of 4 hrs. before injection	7,200	9,500
	5,800	6,500
	4,000	7,000
1 hr. after injection. 3 separate counts	1,000	1,200
	1,400	1,000
	1,600	1,000
3½ hrs. after injection	3,200	6,000
	1,600	5,800
	1,400	5,600
Next morning about 18 hrs. after injection	1,800	16,000
	1,600	24,000
	2,000	32,000
		18,000

The first animal died within twenty-four hours after the injection, and blood cultures taken from the heart's blood before and after death showed numerous staphylococci. The other animal, in which no proteotoxin had been injected with the bacteria, was slightly sick for five or six hours after the injection, but after that it rapidly recovered and remained well.

In order to obtain results as striking as the one presented in the preceding protocol, it is necessary to carry out a considerable number of experiments until the relative doses of proteotoxin and staphylococci are so adjusted that a marked and consistent difference is apparent. For in many cases a too severe dosage with staphylococci, even without the poison, may lead to a considerable prolongation of the stage of preliminary leucocytic depression, as seen in guinea pig 2 after one hour. A too slight dosage with proteotoxin, on the other hand, will result in a temporary leucopenia only and recovery of the animal. Properly adjusted, however, the experiment is instructive in that it shows that the animal receiving the bacteria together with the proteotoxin does not recover from the

preliminary leucopenia which follows the injection, and does not, later, react with the powerful leucocytosis that accompanies recovery in the control animals.

SUMMARY AND CONCLUSIONS.

1. Our experiments show that proteotoxin (anaphylatoxin) produced with typhoid bacilli and active guinea pig serum, by the general method of Friedberger, possesses aggressin-like action in that, when injected together with sublethal doses of bacteria, it renders them lethal.

2. This action is not specific, since even when typhoid bacilli are used in its production, the proteotoxin possesses this property not only for typhoid bacilli, but for staphylococci and prodigiosus bacilli, the only other organisms with which we worked.

3. This action is manifest in the experiment only when quantities of proteotoxin are used which can produce a certain degree of systemic poisoning.

4. It is not due to anti-opsonic action since the phagocytosis of staphylococci in the presence of active serum takes place readily in spite of the presence of relatively considerable amounts of the poison.

5. The aggressin-like action of the proteotoxin is probably due to the leucopenia that it causes. It is not likely that this leucopenia is dependent upon a negative chemotactic action, in the ordinary sense of this term, but it is probably due to the general intoxication of the animal, resulting among other things in the poisoning of those tissue elements which ordinarily react to infection with the mobilization of leucocytes in the circulation.

It has seemed to us that the results of our experiments were of interest also in throwing some light on the general problem of virulence.

The thought naturally suggests itself that Bail's so called aggressins were nothing more or less than proteotoxins, or anaphylatoxin. It has been shown by Friedberger and Nathan (3) that poisons of this description may be formed in the peritoneal cavities of animals, and if we examine the method by which Bail (4) prepared his aggressive exudates, it seems likely that they contained proteotoxins.

Moreover, although Bail's aggressins are described as non-toxic in themselves, this is a discrepancy to which, it seems to us, no great weight need be attached in this connection, since the poisonous properties of the proteotoxins are clearly apparent only when considerable amounts, two to three cubic centimeters, are intravenously injected into young guinea pigs, and below this quantity a threshold is rapidly reached beyond which no visible reaction whatever is achieved. We have not yet, it is true, shown that animals may be immunized against bacterial infection by treatment with the proteotoxins, a result obtained by Bail with his aggressins. But we feel that the mere fact that tolerance to proteotoxin may be established, a phenomenon which has been the subject of an earlier paper, is a step in this direction. Further than this the analogy cannot be carried since we do not know as yet whether the proteotoxin tolerance is specific,—a fact which would be necessary in order to identify completely these substances with the substances studied by Bail. In view, however, of the many objections that have been voiced against this phase of Bail's work, and of the fact that a rapid accumulation of leucocytes is described as characteristic of aggressin immunity, it seems not unlikely that even in this aspect of the problem aggressin and proteotoxin may eventually be identified.

It is probable, also, if we reason logically from the premises which our experiments supply, that the power of invasion of many microörganisms is intimately related to their ability to react with the active blood constituents of the infected animal. This has already been suggested in the work of Embleton and Thiele (5). If, indeed, the condition of bacterial anaphylaxis or hypersusceptibility depends upon the establishment of a balance between the injected bacterial antigen and the blood constituents, such that proteotoxins are formed, a supposition rendered probable by much recent work, then our experiments would tend to show that bacterial anaphylaxis implies, at the same time, a condition of lessened resistance to invasion by the bacteria[1](6). It may even be that generalized inva-

[1] Professor William H. Welch, of Johns Hopkins University, has told us that several years ago, in carrying out some experiments with peptone injections, the animals that received the peptone seemed thereby to be rendered more susceptible to infections. Considering the demonstrated similarity between peptone poisoning and anaphylactic poisoning his observation would correspond to that made by us. We have so far had no opportunity to carry out experiments with peptone.

sion, after localization, must await the striking of such a balance, an interval which, in the case of diseases like typhoid fever, may form a part of the incubation time. At the same time, in such diseases, the action of the proteotoxin would explain the leucopenia that accompanies the bacterial invasion.

We do not, of course, expect to explain virulence in general by these phenomena. In the case of organisms like the pyogenic cocci, the pneumococcus, and some other bacteria, there is apparently another important element contributed in the resistance of the invader to direct reaction with the active serum constituents. In many of these organisms the slight extent or absence of serum lysis or even bactericidal action would point in this direction, and here the appearance of proteotoxin would be secondary in importance or possibly absent entirely until late in severe infections.

The non-specificity of the aggressive action of proteotoxin goes far toward explaining the increased susceptibility to other, secondary infections apparent during the course of many cases of typhoid fever and in some other diseases. It should also have an important bearing on our understanding of mixed infections.

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A METHOD OF COUNTING THE ACTUAL NUMBER OF PURKINJE CELLS PRESENT IN A GIVEN AREA OF CEREBELLUM, AND ITS APPLICATION IN TEN CLINICAL CASES.*[1]

By FREDERIC WADE HITCHINGS, M.D.

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In making extensive studies of nerve cell changes, it was found that while a qualitative method was of certain value, a quantitative method would be essential in order to obtain the greatest possible accuracy. This led to the formation of an arbitrary classification of nerve cell changes (Crile) and to the method of making differential Purkinje cell counts by means of which cell activity could be reduced to percentages of active, fatigued, and exhausted cells. The application of these differential counts has been of wide range, and the more counts that have been made under many carefully checked and counterchecked conditions, the more confidence has been felt in their relative accuracy.

The problem of the exhausted cell as presented by the differential counts is of considerable interest. If we assume that a destroyed nerve cell is never replaced, the problem becomes one of great practical importance. The body can regenerate many of its component cellular parts, but if it cannot regenerate nerve cells, if it has but one supply for the use of the individual during his life, every step taken to conserve this supply would be a step toward maintaining efficiency and probably toward prolonging life itself.

The arbitrary definition of an exhausted cell is that it is one in which, after loss of practically all the intracellular and intranuclear chromatin granules, the nucleolus itself begins to disintegrate. It

* Received for publication, September 21, 1914.

[1] This method was elaborated in the course of the routine work done in the laboratory of Dr. G. W. Crile. Thanks are due to Dr. Crile, to Dr. J. B. Austin for the differential Purkinje cell count figures, and to Dr. H. C. King, for assistance in making the actual Purkinje cell counts.

is not known absolutely that a cell in such a condition cannot recuperate, but there is little doubt but that this is so. Beginning nucleolar disintegration is the first stage of exhaustion. With increasing disintegration or disappearance of the nucleolus the major structural changes occur, from rupture and disappearance of the nucleus down to rupture and disappearance of the entire cell.

The assumption is that an exhausted cell in any stage of exhaustion is a dying cell which cannot be recuperated. What becomes of the exhausted cells? Many observers have found evidence of the removal of nerve cells through phagocytic action, and the consensus of opinion is that the phagocytes are not only derived from the white blood corpuscles but also from the neuroglia cells themselves.

The object of this research is to endeavor to show, by a method of making actual Purkinje cell counts, that the observed facts of cell disappearance are supported by demonstrable numerical losses of the cells themselves. With certain reservations it would be expected that in cerebellar tissue showing very high percentages of exhausted cells there would be a corresponding actual loss of cells. If extreme nerve cell activation were to cause extreme nerve cell exhaustion and death in a short time, it would not be expected that many of the exhausted cells would be removed, but in a severe chronic activation the processes of exhaustion and removal would have time to go on and evidence of removal would be found.

THE METHOD FOR MAKING ACTUAL PURKINJE CELL COUNTS.

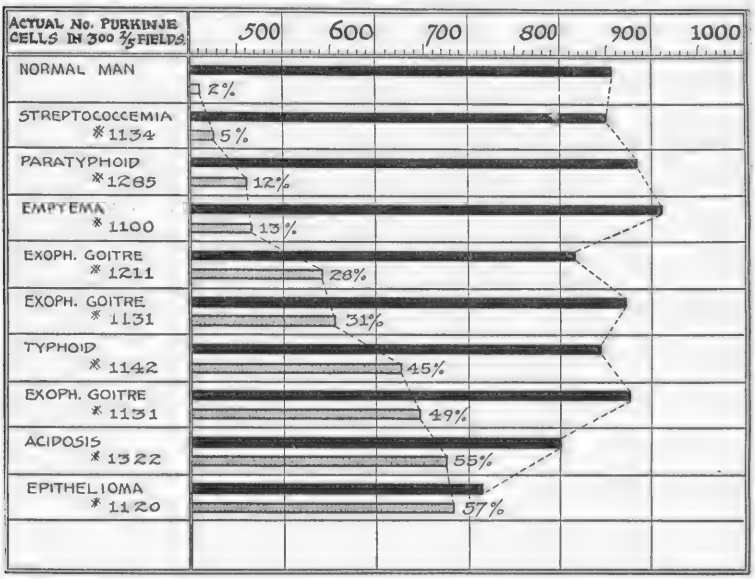
The method consists in counting, with the help of a mechanical stage, the number of Purkinje cells found in 300 fields of the size made by using a No. 2 ocular and No. 5 objective (Zeiss). This can readily be done owing to the linear arrangement of the cells, it being easy to estimate the distance equivalent to the diameter of a field when counting the cells in the angles made by the cerebellar folds. When the cell row is straight it is made to lie in a diameter of the field so as to include as many cells as possible. By following the margins of the cerebellar folds a count begun in one corner of a section can be made to cover the entire section without covering the same area twice. The sections should be of uniform thickness, preferably five microns, and of such a size that an entire count can

be made from one section, or else sections not subjacent should be taken if more than one has to be used.

After counting the cells in the row in a given field the slide is moved exactly one field away, and so on until three hundred fields have been counted, thus covering what is arbitrarily considered to be a large enough area to give a fair idea of the number of cells present, and giving a definite basis for comparison of material from different individuals.

APPLICATION OF THE METHOD.

In applying this method to animals it was found that there was too much variation among normal animals to establish a standard without more labor than could be given at the time (over 100,000 cells were counted). From human brains ten counts were made



TEXT-FIG. 1. The relation between the number of Purkinje cells of all kinds, as shown by actual cell counts, and the number of exhausted Purkinje cells, as shown by differential Purkinje cell counts.

The black lines represent actual counts, while the stippled lines represent the percentages of exhausted cells. Marked actual cell loss is shown only in the cases in which large percentages of exhausted cells were present.

as shown in text-figure 1. The actual Purkinje cell counts are indicated by the solid horizontal lines while the stippled horizontal lines show the percentage of exhausted cells found in the respective differential Purkinje cell counts. The exhausted cell counts are arranged in sequence.

It will be seen that the largest percentage of exhausted cells was in the case of epithelioma and that in this same case the smallest actual number of cells was found (16.6 per cent. less than in the count from the normal man). On the whole, while the curve of actual cell loss does not diminish so evenly nor so markedly as the curve of exhausted cell increase, there is enough convergence to suggest that the underlying principle is correct.

CONCLUSIONS.

1. This method of determining the actual number of Purkinje cells present in a given area of cerebellum is practicable and of sufficient accuracy to make it another useful means of studying nerve cell activity.

2. In its application to clinical cases it is found that increasing nerve cell exhaustion is accompanied by increasing nerve cell disappearance, although it is recognized that theoretically complete nerve cell exhaustion could be present without nerve cell disappearance on account of the individual dying before phagocytic action could take place.

3. This disappearance of nerve cells corroborates the theories and observations made on phagocytosis of nerve cells, inasmuch as it shows that nerve cells disappear from the brain.

4. While there are too few cases to establish a normal actual Purkinje cell count, it is of interest to note that there were 16.6 per cent. fewer cells in the case with the maximum cell exhaustion (57 per cent.) than in the case of the normal man (2 per cent.).

AGGLUTINATION PHENOMENA IN LOBAR PNEUMONIA.*

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Numerous investigators (1 to 11) have noted that when pneumococci are grown in immune serum the organisms exhibit unusual characteristics. Certain observers have also shown that the sera of animals immunized to pneumococci are able to cause agglutination of these organisms when the ordinary agglutination technique is employed.

In 1900 Besançon and Griffon (12) noted the presence of agglutinins in the blood of patients ill of and convalescent from lobar pneumonia. They found that often the homologous strain (the strain derived from the patient) was agglutinated when a heterologous strain was not. This fact suggested that the various strains of pneumococci might possess biological differences which could not be detected by ordinary cultural methods. Eyre and Washbourn (13) and Gargáno and Fattori (14) also noted such differences, but Neufeld and Händel (15) were the first to study thoroughly immunological variations in pneumococci. They worked, however, with relatively few strains of pneumococci.

Dochez (16) and Dochez and Gillespie (17), working with a large number of strains of pneumococci, isolated from patients suffering from lobar pneumonia, were able to differentiate pneumococci into four groups on the basis of cultural and immunological reactions. The three main groups were distinguishable by agglutination and animal protection experiments with a fourth group which includes all strains of pneumococci which cannot be otherwise classified.

More recently Lister (18) in South Africa, approaching the problem from another standpoint, has differentiated into four groups eighteen of twenty strains of pneumococci isolated by puncture of the lungs of patients having lobar pneumonia. This he was able to do by cross agglutination tests with cultures of these strains of pneumococci and the sera derived from these patients at about the time of crisis. Two strains he found to be non-agglutinable.

The present study has been carried out with the purpose of determining the time of appearance and disappearance of agglutinins in the blood of patients suffering from pneumonia, and of learning more concerning the specific character of these agglutinins, especially their specific relation to the various groups of pneumococci.

* Received for publication, October 3, 1914.

METHODS.

Neufeld (19), Jehle (20), Kindborg (21), and others have found that pneumococci are agglutinated by the serum of immunized animals when this is diluted to 1 to 60 or even 1 to 100,000 (21), and that the serum of patients diluted even to 1 to 160 may cause such agglutination when the homologous strains are used. We have found, however, that the reaction is highly specific even as regards the various groups of organisms, so that for the purpose in view the use of diluted serum seemed unnecessary. This is especially true since normal human blood does not contain agglutinins for the pneumococcus, and spontaneous agglutination of pneumococci does not occur with the method employed. Therefore, in making these tests equal parts (0.3 of a cubic centimeter) of a broth culture of the organism to be tested and of the patient's serum were mixed together and macroscopic readings were made after two hours in a water bath at 37° C. and after twenty-four hours in the ice-box. In practically every case agglutination was apparent at the end of the two hour period, if it occurred at all.

The cultures of pneumococci were obtained from culture of the heart's blood of a mouse inoculated intraperitoneally with the patient's washed sputum, or from direct blood culture, or from culture of material obtained by puncture of the lung of the patient.

Certain observers (22) have noted that cultures fresh from the human host are occasionally not agglutinable. Therefore, all cultures were grown for several generations on artificial culture media. To make sure that the cultures employed were agglutinable, they were all tested with antipneumococcus serum specific for groups I and II, before they were used for the agglutination tests with human serum. At each examination the patient's serum was tested against the homologous organism and also against stock cultures belonging to groups I and II.

The blood to be tested was drawn aseptically from an arm vein of the patient. It was allowed to clot, was centrifuged, and the clear serum was pipetted off. The tests were made with serum as fresh as possible, for it has been found that agglutinins in human blood disappear quickly after it is stored. This is especially so if the serum has not been separated from the clot. With highly im-

mune horse serum this is not the case. Serum two years old has been found still to possess strong agglutinative properties.

The correlation between the clinical severity of lobar pneumonia and the bacteriological findings has not only prognostic importance, but also considerable bearing on the effects of serum therapy. It has been learned that infections which are included in groups I and II are usually severe. The serum treatment of lobar pneumonia has been more successful in the cases included in group I than in group II, and experimentally it has been found that antipneumococcus serum I (a serum produced by highly immunizing a horse to pneumococci belonging to group I) possesses greater protective and curative properties than a serum similarly produced by organisms included in group II. It will be noted in the protocol that six of the fatal cases were group II infections, some of which were thoroughly treated with serum.

On the other hand, infections due to organisms in the heterogeneous group (group IV) are almost always mild and the prognosis is favorable. Infections due to *Pneumococcus mucosus* (group III) are often severe, but as this organism under ordinary cultural methods is non-agglutinable, this group may be left out of consideration for the present.

Bearing in mind these apparent differences in the type of disease produced by the different groups of pneumococci, we may make an analysis of the results of the agglutination reactions.

DISCUSSION.

The protocol of all the cases studied is briefly summarized in table I.

TABLE I.

Group.	No. of cases.	Agglutinins present at some time in the disease.	Never present.	Per cent. present.
I	16	16	0	100
II	13	7	6	53.8
III (*mucosus*)	2	0	2	0
IV (heterologous)	9	5	4	55.5

In all, sera from forty cases of lobar pneumonia, due to different types of pneumococcus, were examined. The sera of all the sixteen

cases belonging to group I showed the presence of agglutinins at some stage of the disease. Six of the thirteen cases in group II showed no agglutinins. In five of these negative cases the disease terminated fatally. Repeated examinations of the sera of these patients showed no agglutinins for their own organism or for stock cultures I and II. In all these cases there was no doubt as to the type of the infecting pneumococcus, for four had positive blood cultures and from the fifth an organism belonging to group II was recovered by lung puncture. This confirms the observations of Lister, who was unable to demonstrate the presence of agglutinins in the sera of four cases that terminated fatally.

The impression has been gained that the non-development of agglutinins in the sera of patients with severe infections is evidence of grave prognostic import. Exceptions to this observation have been noted, however, in which a feeble agglutination reaction developed late in two fatal cases. In one of these the patient's serum showed agglutinins on the thirteenth day (the day of admission to the hospital), the patient dying of a complicating meningitis and empyema on the seventeenth day. This case had no serum treatment. In the second case, which was treated with antipneumococcus serum, agglutinins were demonstrable on the thirteenth day of the disease. This patient had four lobes involved and died in extreme dyspnea on the following day.

Observations were made on only two patients infected with *Pneumococcus mucosus*. Hanes (23) has shown that members of this group are specifically agglutinable when treated according to the method of Porges (24), but that they do not agglutinate when subjected to the usual agglutination method. Since the latter method was employed in the present investigation, no agglutinins were demonstrable in the cases recorded.

In four of the nine cases belonging to group IV, no agglutinins were noted in the patients' sera. Two of these negative cases were mild in type, one was quite ill, having a positive blood culture, and the fourth was complicated with influenza. From this last case large numbers of *Bacillus influenzæ* were recovered from the sputum. Unfortunately a lung puncture yielded no culture. It is impossible to say absolutely whether *Bacillus influenzæ* or an atypical pneumo-

coccus or both were the etiological factors. In four of the five cases showing agglutination of the homologous organism, the reaction was feeble and lasted only one day, and in one case, which presented a very severe clinical picture, with positive blood cultures and spreading lesions, the serum developed strong agglutinative power for the patient's own organism on the day of the crisis (ninth day), which power persisted for eighty-four days. This patient later developed an abscess of the opposite lung and resolution of the original lesion was very slow. As the agglutinative power of this patient's serum was so strong and persistent, a few attempts were made to agglutinate with this serum other strains of organisms belonging to group IV, but all with negative results. It has seemed that in this case, as in two others in which agglutinins were demonstrated over a period of 117 days and 112 days respectively, the duration of the lesion (delayed resolution) had some relation to the persistence of agglutinins in the blood. In the other twenty-five cases showing positive agglutination at one time or another, the duration of this phenomenon was relatively short; the longest was twenty-one days, the average about seven days.

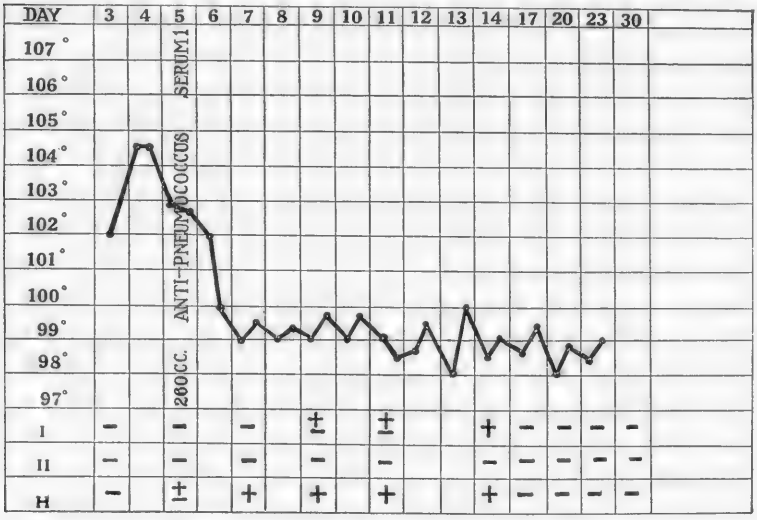
These findings are not wholly in accordance with the results of Jehle's investigations, who found that the sera of only a small number of patients agglutinated the pneumococcus forty-eight hours after the crisis and none after four days.

Agglutinins develop usually just before, at, or just after the crisis (table II, pp. 607–13). During this critical period of the disease Dochez (25) also demonstrated the presence of protective substances in human serum. Only two patients showed agglutinative power any appreciable time before the crisis; one showed it three days before, the second two days before the crisis. The former received antipneumococcus serum, the latter did not.

Too few cases have been studied to draw conclusions as to the effect of serum treatment on the appearance of agglutinins in the patients' blood. Of the sixteen patients in group I, agglutinins appeared in the serum of all. Eleven of these patients were treated with serum, and five received no serum. Of the thirteen cases in group II, agglutinins were demonstrable in the sera of seven cases, and in six cases no agglutinins could be demonstrated at any time.

Eight of the thirteen cases in this group were treated with serum, and of these three developed agglutinins and five did not. On the other hand, four patients showed agglutinins in the blood without serum treatment and none were demonstrated in one fatal case. Thus it would seem that serum treatment had but little influence on the development of agglutinins.

Probably the determination of the presence of agglutinins will have little practical bearing on the diagnosis and treatment of the disease. However, the fact that an organism derived from the



TEXT-FIG. 1. The relative time of development of agglutinins for homologous and stock organisms in a case of lobar pneumonia. I = stock culture of pneumococcus belonging to group I. II = stock culture of pneumococcus belonging to group II. H = homologous culture of pneumococcus, *i. e.*, that derived from the patient.

sputum is agglutinated by the patient's serum is corroborative evidence that this organism is the etiological agent concerned. In practically every case observed, in case the serum agglutinated any organism, it caused agglutination of the organism obtained from culture of the heart's blood of a mouse injected with the patient's washed sputum. Agglutination is of no help in the early recogni-

tion of the type of organism concerned, inasmuch as the appearance of agglutinins occurs relatively late in the disease.

In several instances it has been found that the first organism to be agglutinated was the homologous strain and only later was the stock strain agglutinated. This illustrates the great specificity of the reaction even when no dilutions are used. Clough (26) has noted this marked specificity of the serum of patients recovering from lobar pneumonia in his experiments, demonstrating the power of such sera to render virulent pneumococci phagocytable. One case on which observations were made at frequent intervals shows this group specificity clearly (text-figure 1).

CONCLUSIONS.

1. Agglutinins are present in the blood of patients suffering from lobar pneumonia during some stage of the disease in a large percentage (73.8 per cent.) of the cases due to pneumococci belonging in groups I, II, and IV.

2. In most very severe and fatal cases agglutinins cannot be demonstrated, and it is probable that their absence during the later days of the disease may have unfavorable prognostic significance.

3. No agglutinins are demonstrable by the technique employed in the blood of patients suffering from infection with *Pneumococcus mucosus* (group III).

4. In certain cases agglutinins may be demonstrable for only one day, and in other cases they may persist for several weeks.

5. When agglutinins are demonstrable they usually appear at about the time of the crisis.

6. It has not been possible to demonstrate that treatment with immune serum has any effect on the appearance of agglutinins.

7. The agglutinins present in cases due to organisms of types I and II are always specific for the type of organism causing the infection. In certain cases the agglutination reaction may be more active or appear earlier when the homologous organism is employed than when other organisms of the group are used in the test. In cases due to organisms of type IV, the serum never causes agglutination of any organism except the homologous one.

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TABLE II.

| Case No. | Age in yrs. | | Day of disease. | | |

TABLE II.—*Continued.*

Case No.	Age in yrs.	Day of disease. 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40	Days.	Duration of agglutinins in dys.	Type of organism.	Treatment.	Remarks.
1916	8	S S / I ± / II − / H ± / C	47 / − / − / −	1	I	270 c.c. serum I	Empyema. Recovery.
2021	30	S / I − − − ± + + − / II − − − − − − − / H − ± + + + − / C	9	1	I	260 c.c. serum I	Recovery.
2012	31	I − − + + + / II − − − − − / H − − + + + / C	74 / − / − / −	7	I	0	Recovery.
1978	19	S S / I − − − ± − / II − − − − − / H − − + − − / C	3	1	I	360 c.c. serum I	Recovery.
1939	26	S S S / I − − − + − / II − − − − − / H − − + + − / C	2	1	I	510 c.c. serum I	Died. Pulmonary embolism 16th dy.
1882	22	S S / I − − + − / II − − − − / H − − − − / C	76 / − / − / −	1	I	225 c.c. serum I	Recovery.

Case No.	Age in yrs.	Day of disease																																							Days.	Duration of agglutinins in dys.	Type of organism.	Treatment.	Remarks.
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40					
2083	24	I	..	−	..	..	−	−	+	..	..	+	..	..	−	..	..	..	..	..	..	..	..	..	..	..	..	..	..	±	..	..	..	..	..	..	..	..	..	..	..	..	21	I	0	Recovery.
		II	..	−	..	..	−	−	−	..	..	−	..	..	−	..	..	..	..	..	..	..	..	..	..	..	..	..	..	−	..	..	..	..	..	..	..	..	..	..	..	..				
		H	..	−	..	..	−	−	+	..	..	+	..	..	+	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	+	..	..	..	..	..	..	..	..	..	..	..	..				
		..	..	..	..	C	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..				
2044	38	I	..	..	..	..	−	..	−	..	..	−	..	..	−	..	..	−	..	..	−	..	..	..	..	−	..	..	..	..	..	..	..	−	..	..	..	..	..	..	..	..	9	I	0	Recovery.
		II	..	..	..	..	−	..	−	..	..	−	..	..	−	..	..	−	..	..	−	..	..	..	..	−	..	..	..	..	..	..	..	−	..	..	..	..	..	..	..	..				
		H	..	..	..	..	−	..	−	..	..	+	..	..	+	..	..	−	..	..	±	..	..	..	..	−	..	..	..	..	..	..	..	−	..	..	..	..	..	..	..	..	..				
		..	..	..	..	..	C	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..				
1981	26	I	..	..	..	..	..	..	−	..	..	±	..	+	..	+	..	..	..	..	+	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	10	I	0	Recovery.
		II	..	..	..	..	..	..	−	..	..	−	..	−	..	−	..	..	..	..	−	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..				
		H	..	..	..	..	..	..	−	..	..	−	..	−	..	−	..	..	..	..	−	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..				
		..	..	..	..	..	..	..	C	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..				
		..	..	S	S	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	±	..	..	..	..	..	..	..	..				
		I	−	−	−	..	..	−	..	..	..	−	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	−	..	..	..	..	..	..	..	..	3	I	260 c.c. serum I	Recovery.
		II	−	−	−	..	..	−	..	..	..	−	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	−	..	..	..	..	..	..	..	..				
		H	−	−	±	..	..	+	..	..	..	−	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	−	..	..	..	..	..	..	..	..				
		..	..	..	..	C	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..				
		..	..	..	..	..	..	..	..	S	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	47				
		I	..	..	..	..	..	..	..	−	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	−				
		II	..	..	..	..	..	..	..	−	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	−	0	II	100 c.c. serum II	Recovery.
		H	..	..	..	..	..	..	..	−	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	−				
1880	25	..	..	..	..	..	..	..	..	..	C	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..				
		..	..	S	S	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	129				
		I	..	..	..	..	..	..	..	−	..	..	..	..	−	..	..	..	..	..	..	−	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	−				
		II	..	..	..	..	..	..	..	−	..	..	..	..	+	..	..	..	..	..	..	±	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	−	7	II	175 c.c. serum II	Recovery.
		H	..	..	..	..	..	..	..	−	..	..	..	..	+	..	..	..	..	..	..	+	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	−				
1827	40	..	..	..	C	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..				

TABLE II.—*Continued.*

Case No.	Age in yrs.	Day of disease. 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	Days.	Duration of agglutinins in dys.	Type of organism.	Treatment.	Remarks.
1953	12	I −				−		−		−					−								−			±														−				
		II −				−		−		+					−								−			±														−				
		H −				−		−		−					−								±			−														−	16	II	o	Recovery.
						C																																						
1999	34										−	−		−				−					−		−				−											116				
									−		−	−		−				±					−		±				−											−	8	II	o	Empyema. Recovery.
									−		−	−		−				−					−		±															−				
											−	−													±																			
												C																																
1820	31	I																																										
		II																																										
		H											+						+				+																		10	II	o	Recovery.
							C																																				
—									S	S	S						−																							122				
		I							−	−	−						−									−														−				
		II							−	−	±						+									+														±	112	II	405 c.c. serum II	Recovery.
		H							−	−	±						+									+														−				
1871	33									C																																		
																																									59				
		I −				−				−		−			−			−					−			−														−				
		II −				−				−		+			+			+					+			+														−	20	II	o	Recovery.
		H −				+				+		+			+			−					−			−														−				
1969	19							C																																				
		I								−			−																																
		II								−			−																																
		H								−			−																																
1931	43										Lysis																																		

Case No.	Age in yrs.	Day of disease. 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40	Days.	Duration of agglutinins in dys.	Type of organism.	Treatment.	Remarks.
1876	7	I II H C: 8		1	IV	0	Otitis media. Recovery.
2014	59	I II H: 8 9 10 11 12 C: 11		0	IV	185 c.c. serum I	Delayed resolution. Recovery.
1936	22	I: 11 12 36 40: 46 II: 11 12 36 40: 46 H: 11 12 36 40: 46 Lysis: 7		1	IV	0	Delayed resolution. Recovery.
2032	20	I: 8 11 14 17 25 40 II: 8 11 14 17 25 40 H: 8 11 14 17 25 40 C: 8		0	IV	0	Recovery.
2102	18	I: 6 8 11 12 17 18 25 31 36 40: 46 63 68 93 100 II: 6 8 11 12 17 18 25 31 36 40: 46 63 68 93 100 H: 6 8 9 11 12 17 18 25 31 36 40: 46 63 68 93 100 C: 8		84	IV	0	Abscess of lung. Recovery.
2115	12	I: 6 8 9 11 14 II: 6 8 9 11 14 H: 6 8 9 11 14 C: 8		1	IV	0	Recovery.

TABLE II.—*Concluded.*

Case No.	Age in yrs.	Day of disease.	Duration of agglutinins in dys.	Type of organism.	Treatment.	Remarks.
1857	31	I: 10 +; 24 − II: H: 7 C	1	IV	0	Recovery.
2093	28	I: 5 −; 7 −; 8 −; 9 −; 10 −; 11 −; 13 − II: 5 −; 7 −; 8 −; 9 −; 10 −; 11 −; 13 − H: 5 −; 7 −; 8 −; 9 −; 10 −; 11 −; Lysis	0	IV	0	Recovery.
1904	16	I: 8 −; 10 − II: 8 −; 10 − H: 8 −; 10 −; Lysis	0	IV	0	Recovery.
1930	50	I: 13 +; 16 + II: 13 −; 16 − H: 13 −; 16 −	3	I	0	Meningitis empyema. Died.
1839	37	I: 4 S; 5 S; 6 S; 7 S; 10 − II: 2 −; 3 −; 5 −; 10 − H: 2 −; 3 −; 5 −; 10 −	0	II	1025 c.c. serum II	4 lobes involved. Pericarditis. Died 10th dy.
1856	50	I: 4 S; 5 S; 2 −; 5 − II: 2 −; 5 − H: 2 −; 5 −	0	II	375 c.c. serum II	2 lobes involved. Died 5th dy.

Case No.	Age in yrs.	Day of disease.	Duration of agglutinins in dys.	Type of organism.	Treatment.	Remarks.
1899	62	4 S, 5 S, 6 S; I 4 –, 5 –, 6 –; II 4 –, 5 –, 6 –; H 4 –, 5 –, 6 –	0	II	365 c.c. serum II	Died 6th dy.
1983	45	I 1 –, 4 –, 6 –; II 1 –, 4 –, 6 –; H 1 –, 4 –, 6 –	0	II	0	3 lobes involved. Died 8th dy.
1835	36	4 S, 5 S, 6 S, 7 S; I 4 –, 5 –, 6 –, 7 –; II 4 –, 5 –, 6 –, 7 –; H 3 –, 4 –, 5 –, 6 –, 7 –	0	II	1125 c.c. serum II	4 lobes involved. Died 7th dy.
1858	43	5 S, 6 S, 7 S, 8 S, 9 S, 12 S; I 7 –, 8 –, 13 –, 16 –; II 7 –, 8 –, 13 –, 16 ±; H 7 –, 8 –, 13 –, 16 ±	1	II	1175 c.c. serum II	4 lobes involved. Died 17th dy.
1963	57	I 4 –, 7 –; II 4 –, 7 –; H 4 –, 7 –	0	III	0	Died 7th dy.

CULTIVATION OF THE SKIN EPITHELIUM OF THE ADULT FROG, RANA PIPIENS.*

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Plates 32 to 40.

INTRODUCTION.

It has now been definitely shown, as far as connective tissue is concerned, that tissues and cells when isolated from the organism and cultivated in nutritive media grow actively through division. The cultivated fragments of connective tissue grow not only through cell migration, but also in a literal sense; that is to say, the cells increase in number.

Seventy-eight days after the explantation of cultures of rat connective tissue Lambert and Hanes[1] were able to observe microscopically the various stages in the mitotic division of the individual cells, and Carrel[2] has shown that cultivated connective tissue can be preserved in a state of growth for an indefinite period of time.

Recently Carrel[3] has stated that a strain of connective tissue taken from the heart of a chick embryo and cultivated outside the organism for a period of twenty-eight months possesses a much greater rate of activity than do freshly cultivated fragments of connective tissue. Numerous examples of mitosis in these cultures of more than two years' duration demonstrate the active multiplication of the cells.

The conditions governing the cultivation of epithelial tissue, however, are not equally favorable. The behavior of cultivated epithelial cells has been studied mainly in organs of warm-blooded animals containing epithelial cells. As happens in the case of connective tissue, these epithelium cells shortly after they have been placed into the medium begin to leave the tissue and migrate into the medium, thus forming a membrane of cells around the original fragment. Usually, however, these cells soon perish, and it has not yet been definitely ascertained whether or not they possess the power of actively increasing in

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[1] Lambert, R. A., and Hanes, F. M., Beobachtungen an Gewebskulturen in Vitro, *Virchows Arch. f. path. Anat.*, 1913, ccxi, 89.

[2] Carrel, A., Present Condition of a Strain of Connective Tissue Twenty-Eight Months Old, *Jour. Exper. Med.*, 1914, xx, 1.

[3] Carrel, A., *loc. cit.*

number. Lambert and Hanes,[4] who have made comparative studies of cultures of epithelial and connective tissue cells, have stated that epithelium is much more difficult to cultivate than connective tissue. They have apparently seen no cases of cell division, as no mention is made of this phenomenon.

The results obtained with cultures of epithelial cells of cold-blooded animals were even less satisfactory than those of warm-blooded animals. Hitherto these experiments have been performed only with the epithelium of *Anuræ*. The first cultures of frog skin were made by Ruth[5] in Carrel's laboratory. The object was, however, only to investigate certain processes of cicatrization, for which purpose it was not necessary to keep the frog's skin alive longer than three days. During this time the cultures showed a more or less considerable expansion of the epithelial cells which surrounded the old fragment in the form of a compact layer.

Oppel[6] cultivated the entire tail of tadpoles, and although a membrane emanating from the old epithelium was formed to cover the cut surfaces, he was unable to preserve these objects alive for any considerable length of time, and was thus prevented from following the subsequent fate of the epithelial cells.

The reports of Holmes[7] are nevertheless more satisfactory, inasmuch as they are concerned with the potentiality for life of the cultivated epithelial cells. This investigator worked with embryonic frog skin which he cultivated in the plasma or lymph of the adult frog. The epithelial cells separated themselves from the fragments of skin, both in the form of compact membranes and individually. In the latter case they were subsequently able to unite to form secondary epithelial membranes. In the cases of isolated epithelial cells it was shown that the individual cells can be maintained alive as long as two months. There was no positive evidence of cell division.

Connective tissue and epithelium can be clearly distinguished, at least in the early stages, by the shape of their cells and by their respective modes of expansion in the medium; and Lambert and Hanes[8] have recently reëmphasized the fact that they can readily distinguish the two kinds of tissue in cultures. The connective tissue cells never form compact membranes, but yield a kind of network surrounding the old fragment of tissue in which the cells are oblong and spindle-shaped. The exact opposite obtains in the case of cultivated epithelium; its cells are always more or less polygonal in shape and unite to form compact membranes. Added to this is the fact that connective tissue cells can continue to live and grow for years without difficulty, whereas the epithelium cells perish after a short period of membrane formation.

[4] Lambert, R. A., and Hanes, F. M., *loc. cit.*

[5] Ruth, E. S., Cicatrization of Wounds *in Vitro*, *Jour. Exper. Med.*, 1911, xiii, 422; The Influence of Distilled Water on the Healing of Skin Wounds in the Frog, *ibid.*, p. 559.

[6] Oppel, A., Demonstration der Epithelbewegung im Explantat von Froschlarven, *Anat. Anz.*, 1913, xlv, 173.

[7] Holmes, S. T., Behavior of Ectodermic Epithelium of Tadpoles, when Cultivated in Plasma, *University of California Publications in Zoölogy*, 1913, xi, 155.

[8] Lambert, R. A., and Hanes, F. M., *loc. cit.*

The same morphological differences between the connective and epithelial tissues which exist in the cultures are also found in the organism, in which the two kinds of tissue are strongly differentiated by the above mentioned morphological characters. Whether the same parallel exists between cultivated and normal tissue, as regards the capacity for growth possessed by the two kinds of tissue, and what the causes of this varying capacity for growth are, has not been investigated. This paper is submitted in the hope of contributing in some measure to the solution of this problem.

MATERIAL AND METHOD.

For the purpose of our experiments we selected the leopard frog (*Rana pipiens*), which is easily obtained. We employed as material for cultivation the skin of adult animals, which with the epithelium and cutis was cut into small fragments and placed in the culture medium. The pieces selected for cultivation were usually taken from the back of the frog.

The culture medium was generally composed of a mixture of blood plasma and muscle extract from adult frogs. Great difficulty was experienced from the fact that this mixture coagulates much less easily than some others, than chicken plasma, for example. My observations coincide in many respects with those of Losee and Ebeling[9] in the case of human plasma. The capacity to coagulate possessed by the frog plasma is subject to extreme individual fluctuation, and this is possibly modified further by the seasons. It is probable that this factor is accentuated for a short time following the breeding period.

Semifluid plasma which has not coagulated well is ill suited for the preservation of cultivated tissues for any length of time. For that reason it is of advantage to add a small quantity of chicken plasma to the frog plasma, which effects a satisfactory state of coagulation. Certain plasmas begin by coagulating in a satisfactory manner, but the metabolism of the culture subsequently induces a state of liquefaction. Drops of plasma-muscle extract containing no tissue never liquefy. The same applies to fragments of dead tissue which are only slightly or not at all active. But fragments of skin in full activity give rise within a few hours of explantation to a cavity containing a liquid, even when all the physiological salt solution is carefully removed before placing them in the medium, and they are thoroughly shaken in it before coagulation sets in. The cavity continues to increase the longer the tissue remains in the culture medium, until it attains the dimensions of a drop of liquid. By adding a small amount of chicken plasma to the culture medium the liquefaction can be considerably delayed. The culture medium has to be changed at least every other day, and in cases of liquefaction every day.

[9] Losee, J. R., and Ebeling, A. H., The Cultivation of Human Tissue *in Vitro*, *Jour. Exper. Med.*, 1914, xix, 593.

The cultures of frog skin do not easily support a temperature over 25° C.; temperatures varying between 19° and 21° are more favorable than those above 25° and below 15° C.

EARLY PHASES.

Primary Epithelial Rim.—Soon after explantation changes appear in the cultivated fragment. Figure 1 shows a fragment fixed in formol four hours after removal from the organism and stained by means of Delafield's hematoxylin. Around the fragment of skin, which appears dark in the photograph, a narrow border can be discerned, lighter in color. The latter contains a large quantity of nuclei closely packed together and disposed over each other in several planes. Owing to the density of the tissue the cells belonging to the individual nuclei can be only partially distinguished with the help of considerable magnification, but they are always seen to be polygonal in shape. This primary epithelial layer contains no spindle-shaped cells whatever; the tissue is purely epithelial in character. Certain black cells with branch-like processes which can be distinguished in the deeper strata of the rim of tissue are identical with the small melanophores occurring in the epithelium of frog skin; they have nothing in common with the large melanophores of the cutis and are sharply differentiated from the latter.

Secondary Epithelial Rim.—From sixteen to twenty hours after explantation the cultivated fragments of frog skin are found in a second stage, that of the secondary epithelial rim. This is shown in figure 2. The rim of tissue surrounding the old piece is now seen to have considerably increased in width and its superficial size may even greatly exceed that of the original fragment of tissue. At the same time it has greatly changed in appearance. It now consists of two zones, a narrow central zone and a peripheral zone from five to fifteen times as wide as the former. The central zone represents the upper layers of the primary epithelial rim, which from this time on show no further changes. In the accompanying photograph the central zone appears as a narrow border on three sides of the original piece and is seen to be a little darker than the peripheral zone. As in the case of the primary epithelial rim, this central zone contains small, closely packed nuclei. It is invariably situated above the lighter peripheral zone, so that it looks as if the latter had grown out beyond the central zone.

The peripheral zone is sharply differentiated from the central zone. To begin with, it is lighter and more transparent, owing to the facts (1) that it is composed of fewer layers than the central zone, and (2) that the cells which compose it are flattened out and are hence not so numerous as those constituting the central zone. The character of the tissue is, however, purely epithelial, as appears in figure 2; not a single spindle-shaped cell is to be seen. Nevertheless, the cells composing it are quite different from those of the central zone, as stated above. Each cell can be distinguished individually, and the cell walls are everywhere clearly discernible. The cells are completely flattened, and hence appear large and light in color, and are polygonal in shape. At first they continue to increase in size, as can be ascertained by subjecting the culture to continual observation. The nuclei are large and take the stain very well. They possess one to three nucleoli. Towards the center the peripheral zone consists of

several layers, but at the periphery usually of one only. Immediately behind the peripheral edge a ridge-like zone is often seen, consisting of several layers, the cells of which are closely pressed together. The secondary epithelial rim sometimes contains a very large number of small melanophores, thus showing its epithelial origin. As in the case of the primary epithelial rim, the secondary rim is also nearly uniform; its outer edge is always seen to possess a more or less round, but slightly irregular outline (figure 2).

Loosening Up.—We have seen that during the stages of the primary and secondary epithelial rims the edges of the tissue bordering on the medium are only slightly branched. Everywhere the tissue has the form of a completely compact, continuous membrane. Gradually, however, this picture changes and a progressive loosening up becomes noticeable. Figure 3 shows a culture of another stage to be described later, but it serves to illustrate this process of loosening up. As this process follows the same course in both stages we shall describe it as an example.

First of all, a number of the peripheral cells assume a long, almost spindle-shaped form, as shown in figure 3. Many of them send out long plasma processes into the medium, and these pseudopodia-like processes thus constitute the only link between the cells and the compact parts of the edge of the tissue. They usually assume the form of long chains of cells projecting into the medium, and consist of long, spindle-shaped cells. In this way the line of demarcation of the tissue rim by degrees becomes more and more complicated, and at many places it projects in the form of sharp, jagged protuberances (figure 3).

At the same time spaces are seen to occur in the epithelial membrane, often bridged over by columns of cells. The bridges are also entirely composed of oblong cells, often spindle-shaped. Figure 4 shows a strongly magnified section of the culture seen in figure 3. Most of the cells are still polygonal in shape and their arrangement points to their being strictly epithelial in character. At only one spot where the loosening up process has started can a space be seen in the epithelial membrane, and this is bridged over by a chain of elongated cells.

When the liquefaction of the plasma is very great the loosening up stage assumes a somewhat different aspect. Figure 5 shows a culture which underwent this stage with a rather liquefied plasma. Certain sections, especially those on the right and left sides, where the plasma has remained fairly solid, are seen to have remained compact and to have a very marked fringed edge. In places where great liquefaction has occurred, the membrane has become torn, consequent upon the surface tension of the drop. Large spaces have been formed, partially bridged over by the columns of cells. The cells, following the greatest tension, range themselves at the edge of the drop in the form of a wreath of cells, the columns break down, and their central parts withdraw to the edge of the piece of membrane. If the liquefaction is still more extensive the entire rim of epithelium is torn away from the fragment and a wide wreath of cells is formed, completely surrounding the entire piece of membrane. This wreath at first consists of polygonal and spindle-shaped cells and is connected with the piece of membrane by means of the columns of cells (compare figure 6, slightly magnified, and figure 7, more highly magnified). Later all the columns are broken down by the action of the cells, now spherical in shape (figure 5,

above, central part of the column), which withdraw from the middle of the column towards the periphery and center. The cells constituting the wreath likewise soon become spherical in shape, and the less favorable the conditions of nutrition the more rapidly is this change accomplished, and by the greater number of cells.

Resting Stage.—If the fragment of membrane, together with the surrounding tissue, can be successfully transferred into the fresh plasma, it becomes possible to prolong somewhat the period of the protrusion of chains of cells into the medium. Usually, however, as a result of the liquefaction of the plasma only a narrow rim of cells can be transferred with it into the medium, and owing to the continued loosening up and separation of the individual cells which actively traverse wide portions of the medium, this eventually disappears altogether. Thereafter no additional cells advance from the original culture, and after about seventy-two hours a period of complete rest sets in. From this time on no further changes which might be referred to activity are to be observed, even in portions of the epithelial ring which may yet exist. Only single cells become separated and stray out into the medium; the remainder are still merely subject to change of form which can be attributed to the effect of metabolism. This we shall discuss elsewhere.

Secondary Activity.—The resting stage usually lasts three to four days; namely, from the second to the sixth day after explantation. In all cultures which have not perished through disturbances of nutrition this interval of several days is followed by a period of renewed activity, and the culture passes into the stage of secondary activity. Figure 5 shows a culture during this stage. The culture was made on June 4, and twenty-four hours later had reached the stage of the secondary epithelial rim. Twenty-four hours later, on the morning of June 6, the dissolution was already very much advanced. When the medium was changed almost the entire epithelial rim became dissolved. The following days showed no changes, although the entire piece of skin, as well as the remaining small fragments of the epithelial rim, appeared to be alive and healthy. On the morning of June 8 the medium was again changed, and on the morning of June 9 hardly anything remained of the secondary epithelial rim. On the same day the medium was changed for the third time and shortly afterwards a new rim of epithelium began to develop around the old piece of skin. Numerous identical instances were observed, but we shall confine ourselves to a report of one example.

The tertiary epithelial rim produced by the secondary activity differs but slightly from the first formed secondary epithelial rim. The central zone, corresponding to the primary epithelial rim, which was not concerned in the various changes which have been described, still exists in many cases. It can be made out fairly well in figure 5 as a grey edge surrounding the object. The peripheral zone of the tertiary rim of epithelium is sometimes thinner than that of the secondary rim, and the cells are polygonal in shape and often exceedingly delicate. The small melanophores are either entirely absent or present only in small numbers.

Figure 5 shows furthermore that the tertiary rim of epithelium is subject to the same fate as the secondary. The loosening up of the tissue, the formation of columns and wreaths of cells, and the straying of the cells into the

medium are phenomena which arise as early as twenty-four hours after the first appearance of the tertiary rim of epithelium and lead to a complete breaking up of the tissue membrane.

The behavior of the cultures described above may be considered as typical of the cultures of skin from the back of the adult leopard frog, since these five successive characteristic stages can be observed in most cultures. At the same time it represents the best result that we have thus far been able to obtain with our method. Certain other cultures, however, showed a somewhat different behavior, but it was not always possible to determine the reason of this. Of these exceptions we shall mention one.

In some instances the fragments of skin, after being transferred into the culture medium, remain completely inactive. The first epithelial rim alone is formed and is often extremely narrow. The cultures remain in this condition for some time, although they are treated in exactly the same manner, as far as this can be controlled, as the rest of the active cultures. In the case of culture A 430, for instance, beyond the formation during the first few hours of the first primary epithelial rim not the slightest change appeared in five days, although during this period it was twice supplied with fresh medium. Not until the fifth day, after the third change of medium, did the culture enter upon its active stage. Figure 3 shows this culture on the morning of the sixth day after explantation. By this time the secondary epithelial rim had already reached the loosening up stage (see also figure 4, which represents a fragment of culture A 430 a, highly magnified). The epithelial membrane which, for reasons to be explained later on, consisted only of basal epithelial layers and contained no small melanophores, was extremely thin from the outset. But later, when the loosening up set in and the cells began to project in long strings into the medium it became even more delicate. This can be seen from a comparison of figures 3 and 8; the latter represents the same culture as figure 3, only one day later; *i. e.,* the seventh day after explantation. Moreover, this figure clearly shows the gradual transformation of the polygonal epithelial cells into cells of the spindle-shaped type. Further towards the center where the cells are still united to form a compact membrane, we see everywhere these polygonal forms. Towards the

edge where the cells project into the medium the spindle-shaped type is seen to preponderate.

It should be remarked, with respect to the subsequent fate of culture A 430 a, and of similar cultures which are late in becoming active, that a short time after the period represented in figure 8 the culture had been provided with fresh medium for the fifth time. As a result the entire secondary epithelial rim disappeared. Nevertheless, on the following day, the eighth day after explantation, an equally large rim of epithelium had arisen, which could only be distinguished from the first by its much greater delicacy.

In our discussion of the regular cultures we had reached the fifth stage, that of the secondary activity, and in connection therewith we remarked that the tertiary epithelial rim formed during this stage had suffered dissolution, as in the case of the secondary rim. The original fragment of skin, that is, all which still remained of it, was nevertheless by no means destroyed; on the contrary a new, characteristic stage had developed, the cystic stage. Before discussing this it is necessary to make brief mention of the remarkable change of form undergone by the epithelial cells.

EPITHELIUM AND CONNECTIVE TISSUE.

The tissues of organs consisting of epithelium and connective tissue freshly removed from the body treated by the usual histological methods are seen to be sharply differentiated. Lambert and Hanes[10] emphasize that the same is true of fragments of organs of warm-blooded animals, when cultivated in nutritive media. The epithelial cells are invariably cuboidal, cylindrical, flat, or polygonal in shape, while the connective tissue cells are long and more or less spindle-shaped. Thus, the term "spindle-shaped epithelial cells," as here used, must sound anomalous; and if we furthermore refer to figure 9 as illustrating such spindle-shaped epithelial cells, doubt may arise as to whether the cells are not in reality connective tissue cells. In order to remove this doubt we shall submit some of the facts which serve to prove the epithelial origin of the spindle-shaped cells in the cultures of frog skin.

The fact that no mention has been made of changes occurring

[10] Lambert, R. A., and Hanes, F. M., *loc. cit.*

in the connective tissue, which, of course, was explanted with the epithelium of the skin, may appear remarkable. In none of the skin cultures did the connective tissue of the cutis show the least trace of activity. As the relation between connective tissue and epithelium is in great contrast with the facts ascertained from observation on warm-blooded animals, we have devoted much attention to this point. From the outset the explanted fragments of skin were continually observed, drawn, photographed, and stained. Nowhere could any spindle-shaped cells be detected in the act of separating from the culture. If figure 2, for example, be studied it will be seen that no cells can be discovered bearing any resemblance to connective tissue cells. Many cultures were fixed and stained; in this state they could be viewed from above and below, the magnification could be increased or diminished, but no connective tissue could be discovered. Numerous cultures were examined by this method with unvarying results, as was also the case when they were examined during the earlier stages. Figure 1, representing culture A 429, which had been fixed and stained three hours after explantation, illustrates an early stage. The primary epithelial rim surrounding the fragment of skin shows unmistakable epithelial characters; no spindle-shaped cells can be detected.

The proof of the fact that the membranes composed of polygonal cells surrounding the fragments of skin are in no way connected with the connective tissue lies in the presence in the membranes of small melanophores, which of course are found only in the epithelium and are sharply differentiated from the large melanophores of the cutis.

The continual straying away of the epithelial cells in such large numbers leads finally to the cutis becoming partially freed of epithelium. In this condition the cells can be clearly distinguished in the remaining part, which is composed of connective tissue and is often completely devoid of epithelium; and it can be clearly seen that the connective tissue is definitely and completely circumscribed, and not a single one of its cells has encroached into the surrounding epithelial membrane. So sharply defined is the separation of the connective tissue from the surrounding parts that even the fragment of cutis shrinks into the form of a spherical disc (figure 8).

These facts suffice for the present to prove the epithelial nature of the spindle-shaped cells of cultures of frog skin; later this question will be discussed more fully and further facts will be added.

If the cells shown in figure 9 are epithelial cells, the question arises whether we are further justified in considering the varying form of the cells as a distinct characteristic, which could serve to distinguish connective tissue from epithelium. In the organism another characteristic appears as peculiar to the cell of the connective tissue; namely, the capacity to form fibrillæ. In the tissue cultures, however, in which the connective tissue cells do not always form fibrillæ, we must depend chiefly upon the differences in form of the individual cell species, which fail absolutely in cultures of frog skin. The latter even prove the incontestable fact that the epithelial cells under the special conditions obtaining in the cultures are able to assume the exact form of connective tissue cells. Therefore we must accept the fact that the form of the cells, at least in the tissue cultures, can in no wise be taken to determine whether the origin of a cell is connective tissue or epithelium.

This recalls the reports of cases in which in cultures of organs containing both varieties of cells, *i. e.*, connective tissue and epithelium, the epithelium soon became supplanted by connective tissue, so that ultimately only connective tissue cells remained. The curious changes in the form of the epithelial cells of the frog above described are thus seen to assume an entirely different significance; and it would be a matter of great interest to determine further whether in these instances the epithelial cells are able to assume forms sufficiently distinctive to differentiate them from the connective tissue cells.

DIFFERENCES BETWEEN THE VARIOUS EPITHELIAL LAYERS IN REGARD TO THEIR ACTIVITY.

Not all the layers of the epithelium participate to the same degree in the processes of activity that are observed in a cultivated fragment of skin from the adult frog. The changes occurring in the cultivated fragments of skin depend mainly on the capacity of the epithelial cells to move actively and modify their form. Thus, it is

shown that this capacity is not possessed in the same degree by all the epithelial cells.

The primary epithelial rim (figure 1) needs but a short time for its development. Immediately after explantation into the culture medium, and often while the fragment of skin is yet in the physiological salt solution, this compact rim of epithelium begins to spread over the edges of the fragment. The borders of the primary epithelial rim adjoining the medium are always more or less parallel to the edges of the fragment of skin, by which it is seen that all the cells along one line advance with uniform speed, this being a case of true epithelial movement (Oppel[11]). After a few hours (4 to 24) the epithelial rim becomes more extended; it is still parallel with the cut edges of the epithelial rim, but ceases to be uniform in all the epithelial layers. The cells situated in the deeper layers gradually spread apart to form the peripheral zone of the secondary epithelial rim, whereas the cells of the upper layers completely cease all movement and form the central zone of the secondary epithelial rim. All subsequent changes in the epithelial membrane are confined to the elements of the lower layers, while those of the upper, belonging to the central zone, remain completely inactive and fail to contribute to the formation of the large peripheral zone.

The question now arises: Of what cells are the individual zones composed? This question must be answered if we are to discover what are the specifically active elements of frog skin epithelium. In certain cultures in which the medium was sufficiently firm it was possible to transfer the fragments of skin into the new medium together with the epithelial membrane; but an extremely thin layer became disconnected from the upper surface of the original fragment and was therefore separately placed into a drop of culture medium. A complete, secondary epithelial rim was adherent to the original culture upon transference into the new medium, but the central zone was missing, this having been formed from the separated upper layer. Upon microscopical examination the latter was seen to be composed of several (probably two) layers of large, ex-

[11] Oppel, A., Causal-morphologische Zellenstudien. V. Die aktive Epithelbewegung, ein Faktor beim Gestaltungs- und Erhaltungsgeschehen, *Arch. f. Entwcklngsmechn. d. Organ.*, 1912, xxxv, 371.

tremely transparent, polygonal cells, containing large nuclei and often fine, brownish granules. In figure 10, especially on the left side, these cells can be distinctly seen through the other layers. This membrane was thicker at the edge, at which point the cells were somewhat pressed together. This group of cells corresponded exactly in width to the central zone from which it had been formed at the time of its connection with the culture.

The cell layers concerned in the formation of this upper membrane are obviously the uppermost layers of horny cells of the epithelium. At first the elements constituting them advance into the medium, together with the cells situated beneath them, but this movement soon ceases altogether. If these uppermost layers be isolated from the remainder of the epithelium and maintained in a separate daughter-culture, their cells are seen to possess almost no individual activity at all. They remain alive in the medium for a long time, which fact can be ascertained by means of appropriate nuclear staining. But only the cells situated at the periphery undergo a slight change of form and become somewhat oval. However, this change of shape takes place very slowly. The fact that they nevertheless advance a short distance, in conjunction with the lower layers of the primary epithelial rim, is probably owing to the fact that they are passively carried on by the lower cells; however, as soon as they are disconnected from these cells, all movement ceases.

In many cultures during the stage of the primary epithelial rim a membrane is separated from the upper surface of the culture, which is more compact and consequently darker than the one just mentioned. Upon microscopic examination this membrane was further seen to contain the small melanophores, as shown in figure 11. It consisted of several layers of cells. Figure 10 illustrates the membrane shown in figure 11, seen from below and magnified 370 times. It consists, first, of several layers of small, polygonal cells, among which the small melanophores are distributed, and, second, of the large, flat cells of the uppermost layer mentioned above, which are seen through the layer of melanophores when properly focussed. Thus, while the first mentioned membrane contains only the upper layers, we here have before us the upper and middle epithelial layers, the latter together with the small melanophores. A layer consisting

of such cells could only be separated from cultures which had not yet formed a secondary epithelial rim; but the latter often arose after these layers of skin had become separated, which proves that the basal cells of the epithelium had remained attached to the fragment of skin. However, when the secondary epithelial rim had been formed only after the separation of the upper and middle layers, it naturally had no central zone and was always devoid of small melanophores, as can be seen in figures 3 and 8. But the cells composing it were always typically epithelial.

The membrane of cells consisting of the upper and middle layers of epithelium was separated from the original culture by placing it into separate ones, after which it was maintained in a special daughter-medium. Figure 11 shows one of these membranes three days after isolation. Only a few cells (from the upper edge) are seen to have strayed into the medium, but slight traces of activity could be discerned as late as eight and nine days afterwards. This layer also, when separated from the fragment, was seen to be almost inactive.

It now remains to be shown what attitude is adopted by the basal cells after they have been isolated. To begin with, it could be seen, from the numerous cases in which the secondary epithelial rim was formed only after the separation of the upper and middle layers, that these cells are able to advance actively, even without the co-operation of the upper layers of cells. If a fairly large section be isolated from such a rim of cells and be placed separately in a drop of culture medium, the cells are seen to behave exactly as though they were connected with the whole epithelial rim; they lose their polygonal form and wander out into the medium under the most varied forms, by means of which the entire complex of cells becomes dissolved.

Thus, in the explanted frog skin we are able to distinguish three layers of epithelium physiologically of different value: (1) A basal layer, the cells of which are set in great activity through the conditions prevailing in the cultures; they maintain this activity even when they are kept completely isolated. (2) A middle layer containing the small melanophores, which certainly participates, at least as far as the latter are concerned, in the formation of the

secondary epithelial rim. When, however, it is isolated with the third layer its cells have hardly any power of movement and can advance only slightly into the medium. (3) An uppermost layer whose capacity for expansion is confined to the formation of a narrow rim and disappears altogether upon isolation.

It was not possible at this stage to ascertain whether this physiological differentiation into three layers of the epithelium of the frog's skin coincided with the morphological differentiation, which similarly shows three layers. It is certain, however, that the differences between the individual layers of shifting epithelium are of great significance for a number of normal and pathological morphogenetic processes, for which reason we desire to give special attention to this fact in our subsequent investigations.

Thus, the formation of the secondary epithelial rim is mainly attributable to the activity of a basal layer of cells; at first these cells move as compact membranes into the medium, later on they become separated, the epithelial cells which were hitherto polygonal in shape become spindle-shaped, and after having become isolated they move like connective tissue cells.

From this we see that the epithelial cells are by no means compelled to assume the epithelial movement which Oppel has designated as characterizing the epithelium; this occurs only as long as they form compact membranes. If they become very much separated from each other the manner of their movement is the same as that of connective tissue cells. Not alone with regard to their form, but also through their manner of movement the epithelial cells are capable under special circumstances of approaching the type of the connective tissue cells. It is interesting to note in this connection that Holmes[12] saw a movement of the epithelial cells caused by pseudopodia in the explanted skin of young larvæ of *Anuræ*.

THE EPITHELIAL CYST.

It has been shown that in the explanted fragment of skin stages of activity alternate with stages of rest. Thus far it has not been possible either to maintain the cultures in continuous activity or to induce a state of activity more than twice. In the active stage a

[12] Holmes, S. T., *loc. cit.*

rim of epithelium of a relatively large area is developed each time around the fragment, after which exhaustion occurs. But the fragment of skin by no means dies; on the contrary it undergoes a series of extremely interesting changes, which bear witness to its undiminished vitality. Even as early as the stage of secondary activity, *i. e.*, from the sixth to the seventh day, the fragment of skin, which was originally square, becomes drawn into the form of a spherical disc whose edges tend to bend down. This contraction of the tissue continues until we finally see it in the shape of a sphere. At first it is pigmented only on the upper surface, while the under surface is whitish in color and resembles closely an amphibian egg. By degrees, however, the pigment spreads to the under surface until finally the whole sphere is brownish in color.

The uppermost layer is usually discarded a few days after explantation, and frequently the middle and upper layers of the epithelium have also become separated; but in spite of this fact a loose clinging layer is soon observed around the sphere, consisting of more or less polygonal and usually rather oblong cells. This layer becomes detached in the course of one of the transplantations into the new medium, whereupon a new one is immediately formed, which may also become detached. In this manner a fragment of skin discards three layers of cells in the course of twenty days, besides which twice during the active stage it sent a considerable number of epithelial cells into the medium. Figure 12 represents one of these cysts, aged eighteen days, and likewise shows a layer in the act of becoming detached, which can be seen particularly clearly on the lower surface.

The cell membranes which have been discarded during the cyst stage consist in part of dead, nucleus-free cells, and in part of living cells of the same form as those constituting the uppermost epithelial layers, and, like the latter, contain in their plasma fine, brownish granules. The nuclei are easily stained. If the first epithelial layer consists of horny cells, as is generally assumed, this can also, on account of the absolute identity of the cells, be taken as being the layer of the cells which have been discarded during the cyst stage. Thus, we are here concerned with a process of shedding.

In spite of the repeated and extensive loss of epithelium, the sphere, which is filled with well preserved connective tissue (as can be seen in sections), still possesses a considerable covering of epithelium. This is seen to be differentiated into separate layers of cells. The innermost layer consists of cuboidal cells, which supposedly correspond to the basal cells. Next to these is a layer which usually consists of two rows of cells which are flattened, and the nuclei of which together with the long axis are parallel to the upper surface. The nuclei of both layers stain well. At the exterior is another peel-like layer of extremely flattened cells, which are separated from the two inner layers of cells by means of a small space, the cells of which are only loosely connected. Small melanophores are found in none of the three layers of cells.

From the sections it can be seen that not only the epithelium, but also the stratum spongiosum of the cutis has completely enveloped the stratum compactum and has assumed a spherical form. This explains the complete pigmentation of the cyst.

We may now inquire whence is derived the inexhaustible supply of epithelial cells. Unfortunately, it has not been possible thus far to answer this question. In the first place, it is inconceivable that the original epithelium could repeatedly give off such relatively large masses of cells without undergoing multiplication; but, on the other hand, we nowhere see any trace of mitosis. In the cysts the epithelium shows a distinct differentiation into a basal layer of cubic cells and into a second layer lying above it, consisting of several layers of flat cells. But the basal cells are nowhere found in a state of mitosis.

Thus, for the present, we must content ourselves with establishing the fact that the explanted fragments of skin are gradually transformed into a spherical form, completely shut off towards the exterior, containing a compact connective tissue core and an epithelial coating. In this form the cultures of skin remain alive for a long time and even show the process of shedding which characterizes normal epithelium.

MECHANISM OF THE EPITHELIAL GROWTH.

The life cycle of a culture of skin may serve to throw some light on the mechanism of epithelial growth.

As mentioned above, it is usually much easier to cultivate connective tissue than epithelium in culture media. The conditions created through life *in vitro* are much more favorable for connective tissue than for epithelium, for the reason that explantation modifies to a much less extent the normal manner of growth of the former than of the latter.

In cover-glass cultures the tissues are surrounded by the medium equally on all sides; this practically corresponds to the conditions under which connective tissue lives in the organism under normal conditions, where it is always surrounded by the body juices.

Craster[13] has proved in the case of the epithelium of rat skin that through subcutaneous transplantation of fragments of skin the conditions become so changed, owing to their transference to the interior of the body, that in a very short time the epithelium entirely lacks the power of resuming its normal function when transferred back to its original position. In cover-glass cultures the fragments of skin are subjected to similar abnormal conditions, for they are excluded on all sides from the external world by means of the medium. But it is a characteristic of all epithelia that they possess not only a basal, but also a free surface; they are nourished from the former exclusively, whilst the free surface is differentiated to fulfill special functions. Thus, all epithelial cells, as well as the entire epithelium, are always differentiated as a whole along a polar axis which is perpendicular to the surface of the organ. The connective tissue cells grow freely in the organism in all directions, whilst the epithelial cells under normal conditions grow almost exclusively in a vertical direction; *i. e.*, along an axis perpendicular to the surface of the organ. Under abnormal conditions, as in the case of regeneration, this growth is parallel to the surface of the organ; the cells, however, never grow freely, but along the surface of the organ.

While the connective tissue is able to develop nearly all its characteristics normally in the medium, the explanted skin epithelium is confronted by entirely different conditions. The isolation of the fragment of skin and the consequent formation of free wound surfaces immediately give rise to regeneration, as would also have

[13] Craster, C. V., Conditions Governing the Growth of Displaced Normal Tissue, *Jour. Exper. Med.*, 1912, xvi, 493.

occurred in the organism. The enormous expansion of the epithelial cells into a surface parallel to the upper surface of the fragment of skin corresponds in every particular to the behavior of the skin epithelium in the organism when stimulated by incisive wounds. However, it continues to spread along the surface of the organ deprived of epithelium and the epithelial cells thus suffer either no suspension at all of their polar differentiation or only a passing suspension. But in the plasma cultures the epithelial cells stray freely into the medium, thus losing ground beneath them, and by this means they enter upon physical conditions similar to those of normal connective tissue. They therefore abandon their polar differentiation and take on the morphological character also of connective tissue cells. Their compact epithelial arrangement vanishes and the cells become spindle-shaped. Not meeting with any impediments tending to terminate their movement as under normal conditions, they continue to wander away from the explanted fragment of skin, and the epithelial rims are finally reduced to complete dissolution.

This never ending spreading out of cells is not terminated in the cultures by means of contact stimulus, as happens under normal conditions. Thus, in spite of the fact that a considerable number of living epithelial cells remain, the activity of the fragment of skin becomes exhausted, and the formation of epithelial rims which glide out into the medium becomes suspended. Meanwhile, an increasing contraction of the explanted fragment of skin has already taken place, and the latter soon becomes spherical in form. Fragments of skin which were examined during this stage were already found to be completely surrounded by epithelium. But the fact is by no means excluded that this surrounding of the fragment of skin was initiated immediately after explantation, and that it is owing to this that the connective tissue cells are prevented from straying into the medium and that the contraction of the fragments of skin is brought about. We shall later on institute more detailed investigations with regard to this point.

The epithelium is thus brought into a condition much more nearly approaching normal. The growth which at first was parallel to the upper surface naturally ceases the moment that the edges of the epithelium, which on both sides are growing around the connective

tissue core, come in contact with each other, thus forming a hollow sphere. The conditions prevailing on the inner and outer sides, respectively, of the epithelium are now different, as happens also under normal conditions. Although the outer surface is not in contact with water or damp air, as would be normal in the case of frog skin epithelium, there are present nevertheless a free and a basal surface, the latter in organic combination with the base. Thus, here again we are presented with a case of distinct polar differentiation of the skin epithelium, which is manifested by the presence of three layers.

But furthermore, the process of shedding, which under normal conditions represents the result of vertical growth and goes hand in hand with the replacement of cells originating from the basal layers, is reintroduced, thus completing the resemblance between explanted and normal epithelium. It is true that so far we have not succeeded in discovering any examples of mitosis in the basal cells; but inasmuch as only very few of the epithelial spheres have so far been examined in sections, we must refrain from stating positively by what means the giving off of very considerable numbers of cells is rendered possible.

SUMMARY.

1. Fragments of skin taken from the back of the leopard frog were cultivated in a mixture of plasma and muscle extract of the same species.

2. A few hours after explantation, processes of activity are seen to arise, which finally lead to the formation of a compact epithelial rim around the fragment of skin.

3. These epithelial cells undergo gradual transformation into a spindle-shaped type of cell; in this form they resemble the spindle cells which have been described as connective tissue cells.

4. The growth of a rim of tissue around the explanted fragments of skin may be ascribed principally to the activity of a basal layer of epithelial cells, the units of which first advance into the medium as compact membranes by means of the so called epithelial movement, but which later become separated from the compact membrane, and having assumed a spindle form, spread according to the manner of connective tissue cells.

5. In contradistinction to this basal layer, the cells of an upper and middle layer of epithelium upon separation from the fragment of skin and isolation in the nutritive medium, remain completely inactive.

6. The connective tissue of frog skin, for reasons not yet completely established, does not participate in the production of the rim of cells, possibly for the reason that it was early surrounded by the epithelium and was thus prevented from sending out cells into the medium.

7. The transformation of the epithelial cells into a type of cells of the spindle form is a result of the changes in the physicochemical conditions brought about by the life *in vitro,* which become similar to the physicochemical conditions normally characteristic of connective tissue.

8. The final stage of the explanted fragments of skin is a hollow epithelial sphere (cystic stage) filled with connective tissue.

9. During the cystic stage the epithelium again shows its normal polar differentiation, as a consequence of the physicochemical conditions which now approach normal.

10. These conditions also permit of the shedding of cells in a vertical direction, although previously the production of cells in a horizontal direction had already become impossible.

EXPLANATION OF PLATES.

Plate 32.

Fig. 1. Primary epithelial rim. A rim of pure epithelial tissue surrounding the original fragment of skin. The small dots are cell nuclei, the large black specks in the epithelial rim (especially clear on the lower part of the figure) are the small melanophores of the epithelium. Photograph 20, culture A 429; stained with hematoxylin; magnification, 60; three hours after explantation.

Fig. 2. Secondary epithelial rim. The epithelial character of the tissue (polygonal form of the cells) can be clearly discerned, as well as the large cell nuclei. The small, black specks which are sometimes extended into long processes are the small melanophores. On three sides of the original fragment the central zone of the secondary epithelial rim can be seen. It is a little darker than the peripheral zone. Photograph 22, culture B 13; photographed while alive; forty-eight hours after explantation into a medium composed of frog plasma, chicken plasma, and frog muscle extract.

PLATE 33.

FIG. 3. Secondary epithelial rim, after the uppermost and middle layers of epithelium have been discarded. As the middle layer with the small melanophores was absent during the growth of the secondary epithelial rim, the small melanophores are absent in the latter. Moreover, the figure also shows the beginning of the loosening up process; jagged processes consisting of long drawn out cells are entering the medium. Photograph 2, culture A 430 a; photographed while alive; magnification, 80; six days after explantation.

FIG. 4. Loosening up. This shows a greatly magnified section of the rim of the culture illustrated in figure 3. In the epithelial skin consisting of polygonal cells a gap can be seen spanned by a long drawn out cell. Photograph 3, culture A 430 a; photographed while alive; magnification, 350; six days after explantation.

PLATE 34.

FIG. 5. Secondary activity. The tertiary epithelial rim, formed after the dissolution of the secondary epithelial rim, is here likewise in process of dissolution. But on the left and right the epithelial structure can still be clearly made out. On the other hand, rings of cells have formed above and below, which are partially connected with the original fragment by means of columns of cells. One of them (above) has just become torn; the cells at the center have become spherical in form. Photograph 1, culture A 406; photographed while alive; six days after explantation.

PLATE 35.

FIG. 6. Loosening up in very fluid plasma. A wide ring of cells surrounds the fragment, with which it is connected by means of columns of cells. Culture A 405; drawn while alive; magnification, Leitz ocular 1 + objective 3; thirty hours after explantation.

PLATE 36.

FIG. 7. Columns of cells of a culture undergoing loosening up. Culture A 126; drawn while alive; magnification, Leitz ocular 1 + objective 6; twenty-four hours after explantation.

PLATE 37.

FIG. 8. Loosening up. The same culture as in figure 3, one day later. In several places the transformation of the epithelial skin into protuberances consisting of long spindle-shaped cells can clearly be seen. Photograph 7, culture A 430 a; photographed while alive; magnification, 60; seven days after explantation.

PLATE 38.

FIG. 9. Spindle-shaped epithelial cells. Taken from a culture in the process of loosening up. Photograph 19, culture A 214; stained with hematoxylin; magnification, 350; forty-eight hours after explantation.

PLATE 39.

FIG. 10. Figure 11, more highly magnified. The large polygonal cells of the uppermost layer can be seen through the middle layer. Photograph 5 a, culture A 431 b; photographed while alive; magnification, 370; three days after isolation.

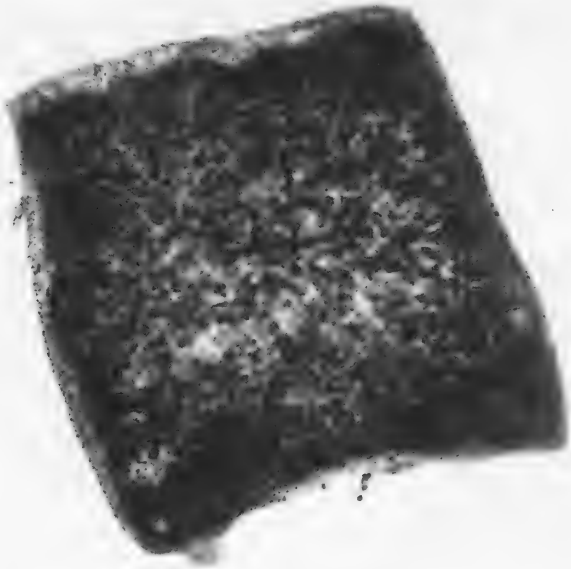

FIG. 1.

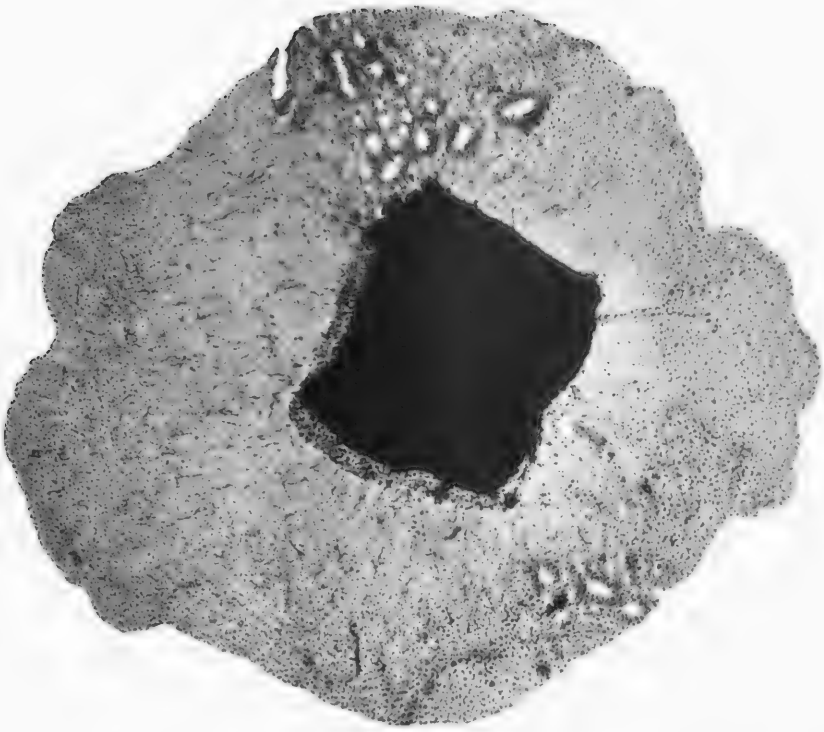

FIG. 2.

(Uhlenhuth: Cultivation of Skin Epithelium of Frog.)

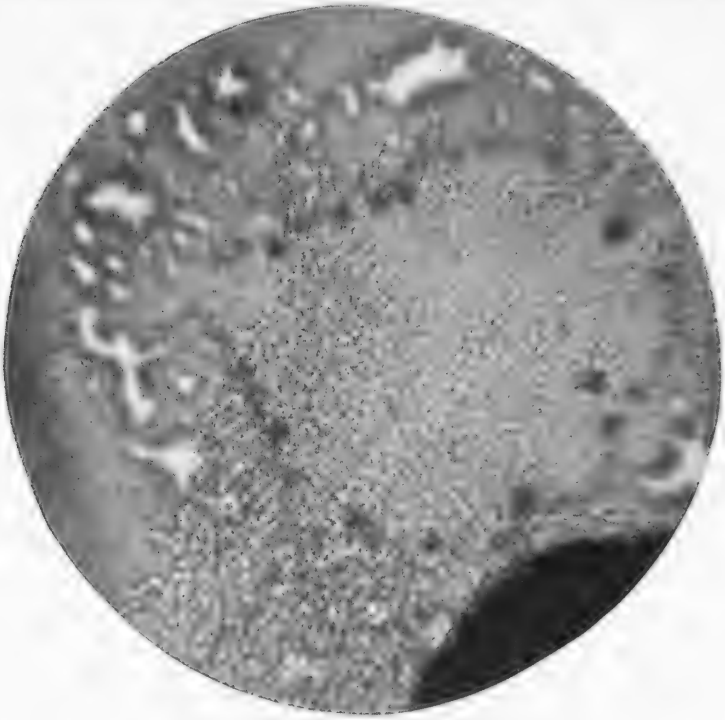

FIG. 3.

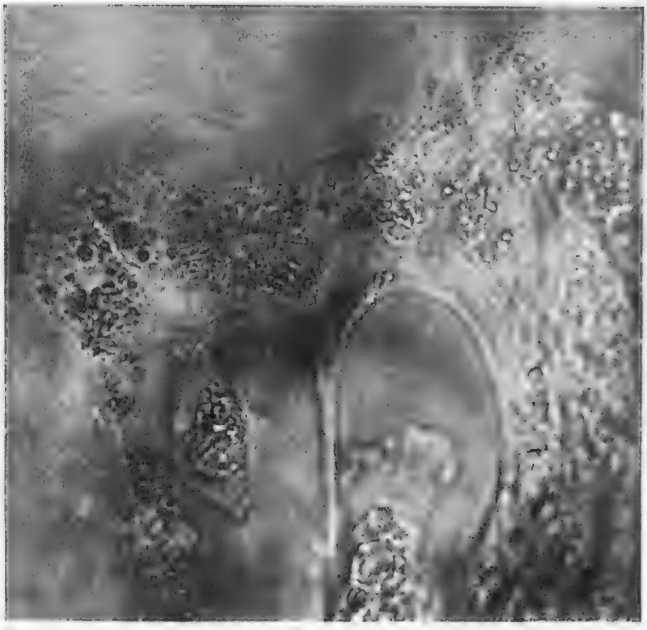

FIG. 4.

(Uhlenhuth: Cultivation of Skin Epithelium of Frog.)

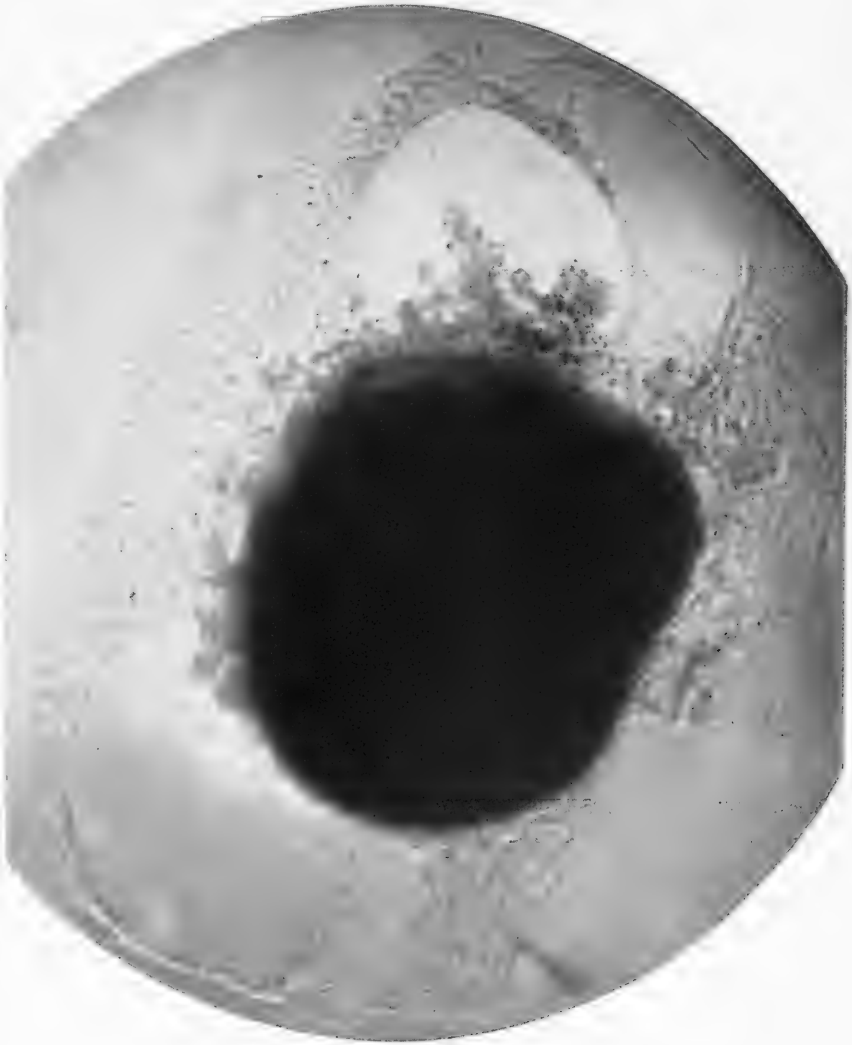

FIG. 5.

(Uhlenhuth: Cultivation of Skin Epithelium of Frog.)

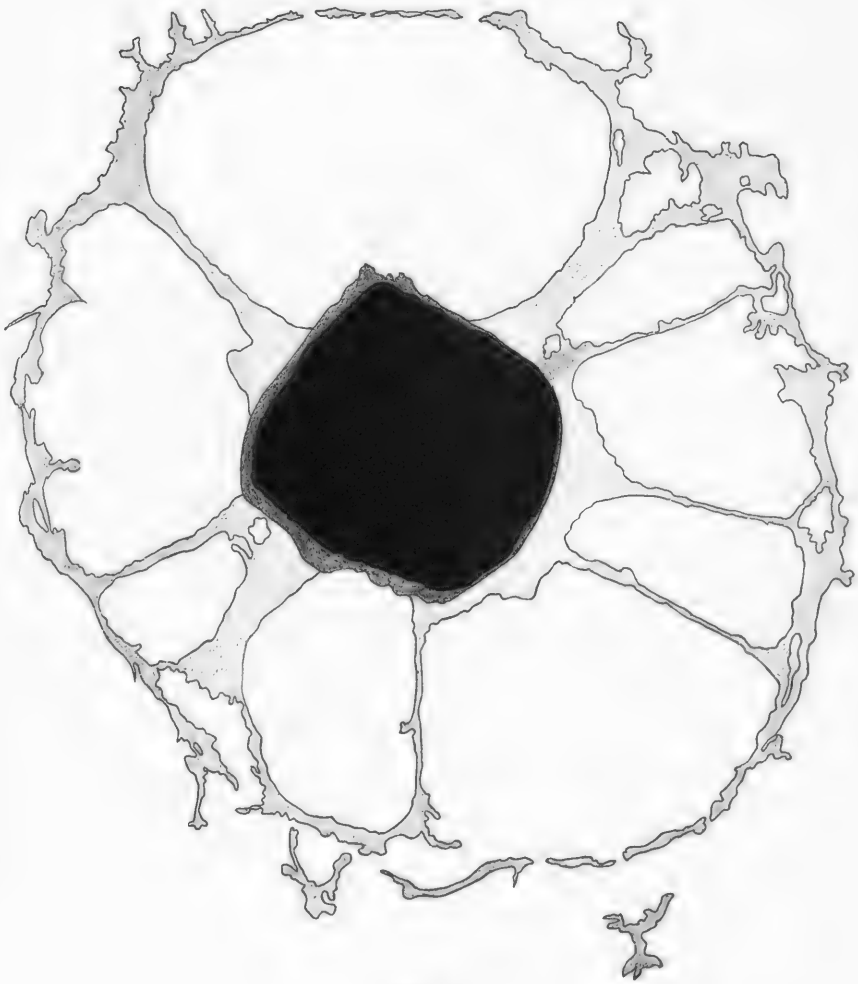

FIG. 6.

(Uhlenhuth: Cultivation of Skin Epithelium of Frog.)

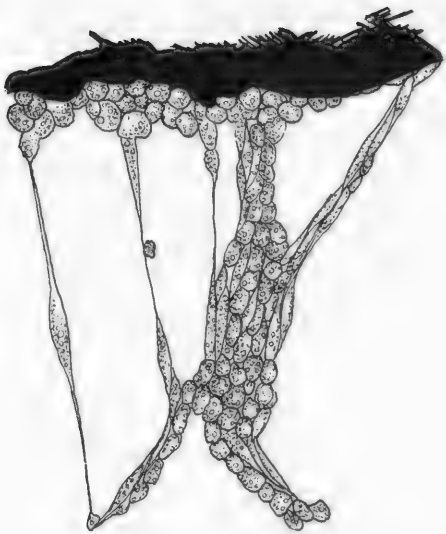

FIG. 7.

(Uhlenhuth: Cultivation of Skin Epithelium of Frog.)

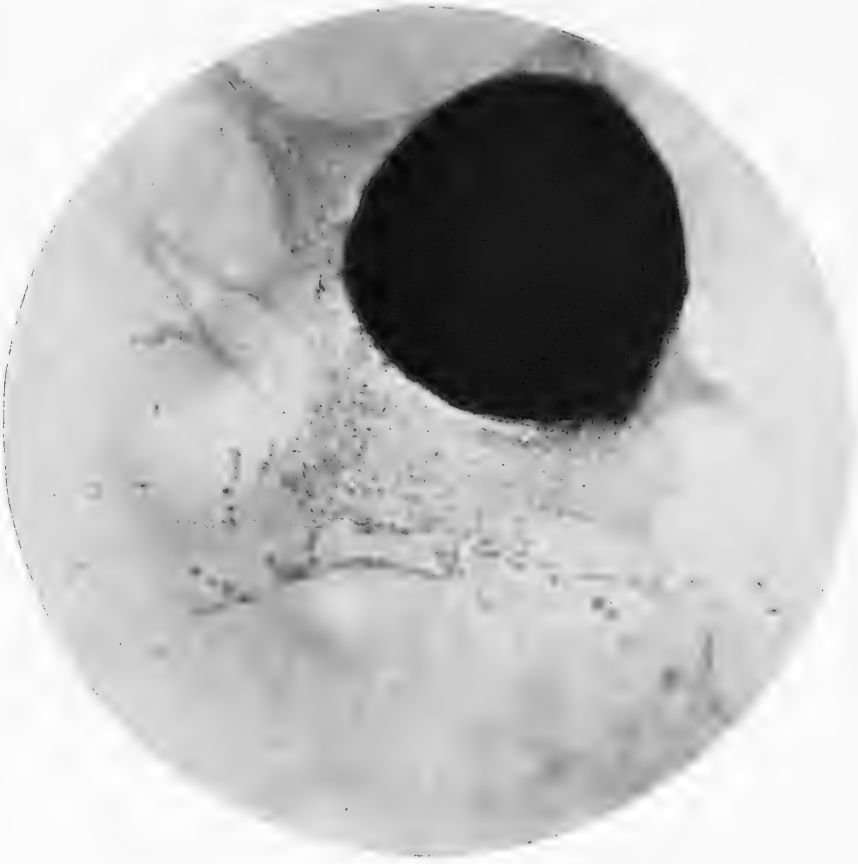

FIG. 8.

(Uhlenhuth: Cultivation of Skin Epithelium of Frog.)

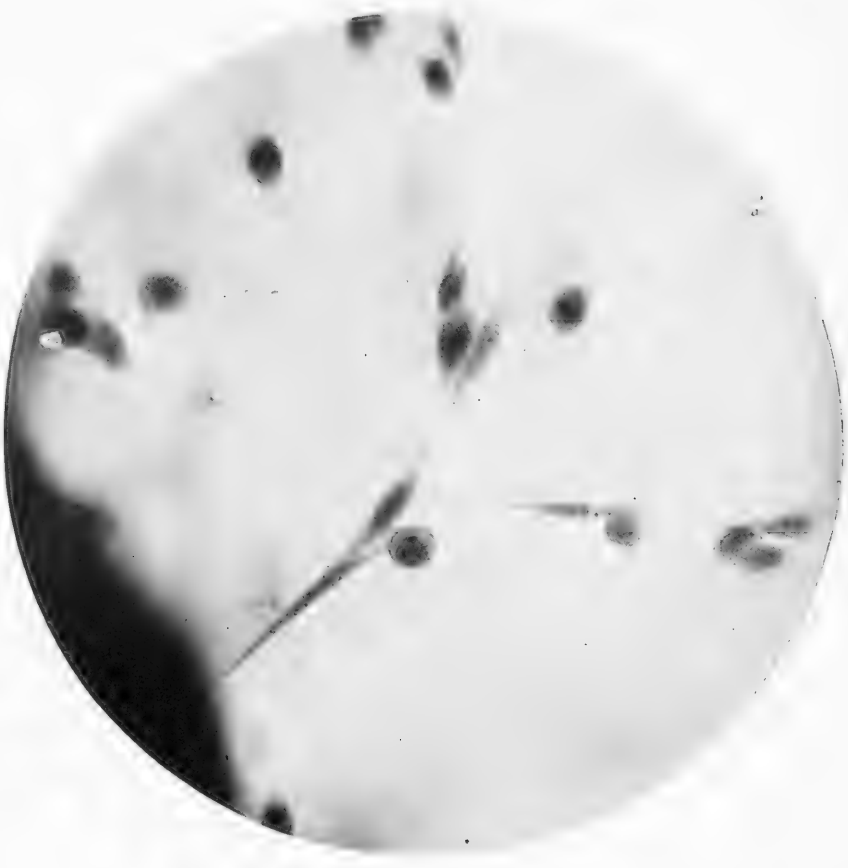

FIG. 9.

(Uhlenhuth: Cultivation of Skin Epithelium of Frog.)

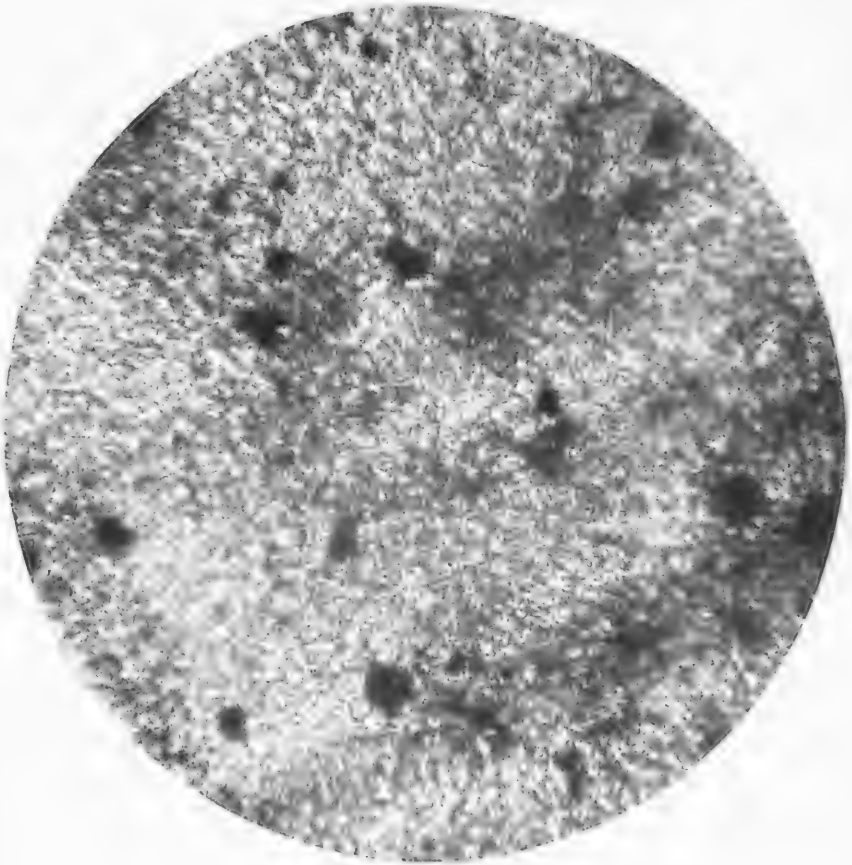

FIG. 10.

(Uhlenhuth: Cultivation of Skin Epithelium of Frog.)

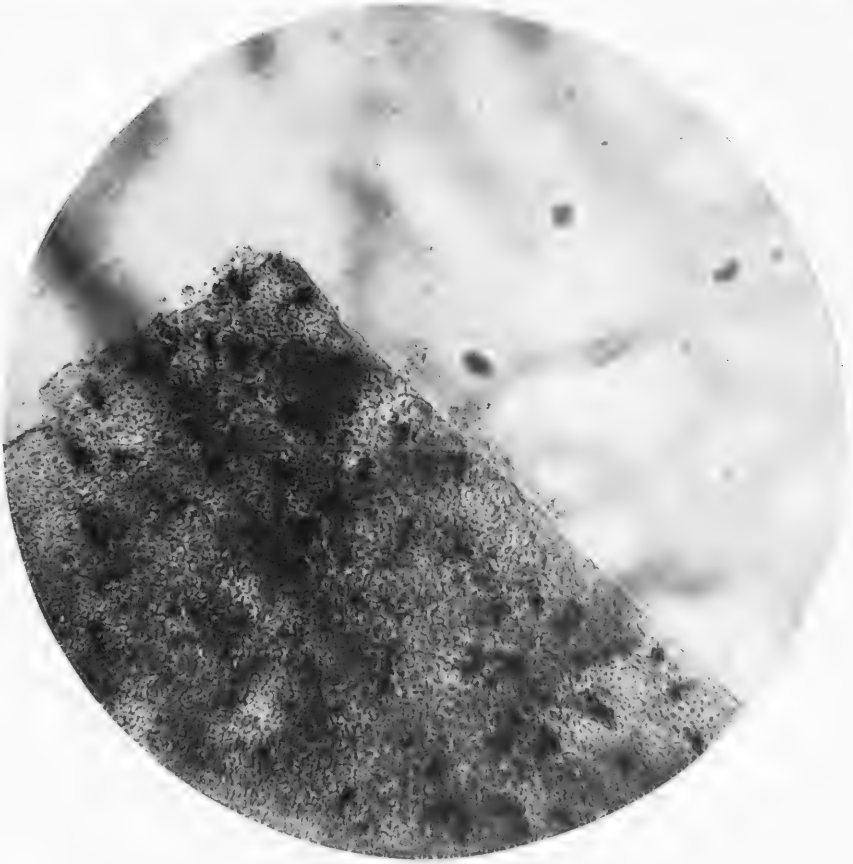

FIG. 11.

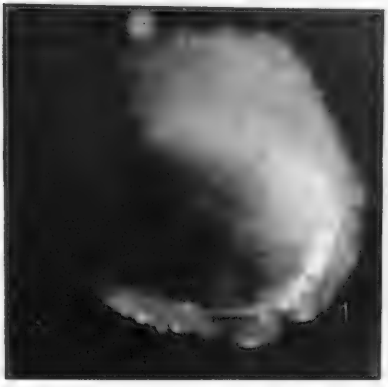

FIG. 12.

(Uhlenhuth: Cultivation of Skin Epithelium of Frog.)

PLATE 40.

FIG. 11. Uppermost and middle layers of epithelium, separated from the explanted fragment of skin and viewed from beneath. The small, polygonal cells of the middle layer can be seen, as well as the greatly contracted small melanophores, which appear as small specks. On the upper edge some cells have advanced into the medium, but all the other cells have remained inactive. Photograph 4, culture A 431 b; photographed while alive; magnification, 120; three days after isolation.

FIG. 12. Epithelial cyst. At the lowest rim of the sphere originating from the explanted fragment of skin a layer consisting of cells in the act of becoming detached can be discerned. Photograph 10, culture A 406 d; photographed while alive; slight magnification; eighteen days after explantation.

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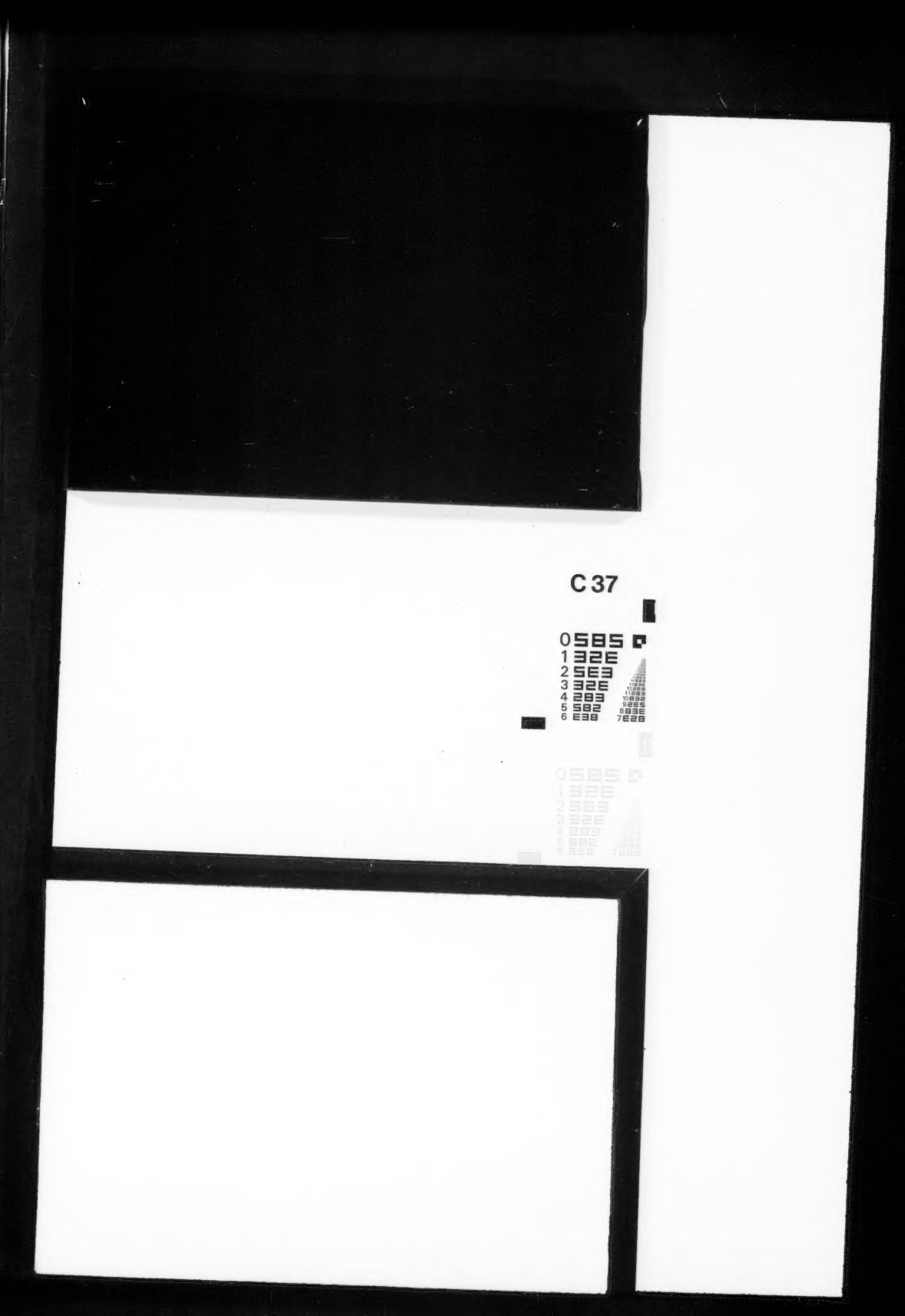
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